THE UNIVERSITY OF ALBERTA

SYNTHESIS OF NITROSOUREAS AND THE STUDY OF THEIR CHEMICAL REACTIONS WITH NUCLEIC ACIDS

by

© LARRY W. McLAUGHLIN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SYNTHESIS OF NITROSOUREAS AND THE STUDY OF THEIR CHEMICAL REACTIONS WITH NUCLEIC ACIDS.

submitted by LARRY W. McLAUGHLIN

in partial fulfilment of the requirements for the degree of Doctor of PHILOSOPHY.

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ABSTRACT

Various aspects of the chemistry of active antitumor 2-haloethylnitrosoureas have been examined in this dissertation. The rates of nitrosourea decomposition in physiologically buffered solutions to produce reactive electrophiles have been measured polarographically. The products resulting from the decompositions suggested a number of possible reactive intermediates. A 2-choroethyl diazohydroxide, cyclic chloronium ion, 1,2,3-oxadiazoline and 2-imino-N-nitrosooxazolidinone have all been examined as potential reactive species.

2-Haloethylnitrosoureas have been observed to alkylate and produce interstrand cross-links in DNA. The cross-linking was observed to increase with increasing pH in the range 4-11, increasing G + C content of natural DNA and was most marked for chloroethyl derivatives. Two chloroethylcytosine model compounds were observed to retain residual alkylating activity and implicate analogous modified cytidine residues in DNA to explain the cross-linking phenomenon.

Nitrosoureas were also observed to produce DNA single strand scission (SSS) of two types. Type I SSS is most extensive for 2-hydroxyethylnitrosoureas and appears to result from the formation of DNA phosphotriesters. Type II SSS results from base alkylation followed by depurination or depyrimidination. The labile apurinic

site was observed to be converted to a single strand break either enzymatically, by high pH conditions or by reaction with an appropriate amine.

The correlation of antileukemic activity with DNA cross-linking initiated the design of new compounds considering five aspects: (i) Compounds which have shorter half-lives, (ii) compounds with leaving groups superior to chlorine, (iii) related nitrosothioureas, (iv) chloroethyl alkylating agents from other compounds and (v) compounds in which the alkylating portion of the molecule was modified. Cross-linking ability and *in vivo* activity were compared when possible.

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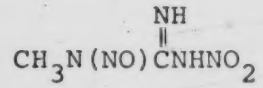
CHAPTER ONE

INTRODUCTION

An investigation into the mechanisms by which nitrosoureas interact with cellular constituents must by its nature involve a number of research areas. This study attempts to detail the chemical mechanisms involved in the decomposition and subsequent interactions of nitrosoureas with purified DNA. However, prior to reporting the results of this dissertation, a brief introduction concerning previous studies of the molecular mechanisms of the biological, biochemical and chemical effects of nitrosoureas will be presented.

The Cancer Chemotherapy National Service Center has routinely screened a wide variety of compounds for therapeutic activity against murine leukemia L1210. In the early 1960's the activity shown by N-methyl-N'-nitro-N-nitrosoguanidines 1[1] stimulated further investigation[1-4] of compounds which might result in diazoalkane alkylating



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agents. Initial studies of the nitrosoguanidine took place at Stanford Research Institute[1,2] with the related nitrosoureas investigated at Southern Research Institute.[3,4] It was soon observed[5] that intraperitoneal injection of

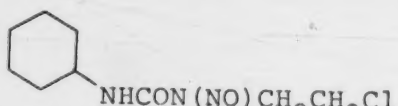
of N-methyl-N-nitrosourea 2, in contrast to the nitrosoguanidines and many other typical chemotherapeutic alkylating agents, resulted in activity against intracerebrally inoculated leukemia L1210 cells. Structure modification of N-methyl-N-nitrosourea 2 produced 1-(2-chloroethyl)-1-nitrosourea 3 and 1-(2-fluoroethyl)-1-nitrosourea 4 with significantly enhanced activity.[3,4]

$ClCH_2CH_2N(NO)CONH_2$ — 3

$FCH_2CH_2N(NO)CONH_2$ — 4

Many such analogues containing the 2-chloroethyl moiety and nitroso function on the N-1, and a wide variety of alkyl, aromatic and heterocyclic substituents on the N-3 positions of the urea have since been prepared for *in vivo* testing.[3,4,6] Two of the most active derivatives, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) 5 and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) 6 have recently been released for commercial preparation and clinical distribution under the trade names carmustine and lomustine, respectively.

$ClCH_2CH_2N(NO)CONHCH_2CH_2Cl$ — 5



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The biological activity of the nitrosoureas and nitrosoguanidines is thought to result from their

decomposition under physiological conditions without enzymatic activation to produce diazoalkane alkylating agents and isocyanates.[7] Previous work involving the relationships of the chemical properties, lipophilicity, alkylating activity and carbamoylating properties, to therapeutic usefulness resulted in the suggestions[5,8-10] that the alkylating portion of the nitrosourea is responsible for therapeutic effects, the isocyanate involved in carbamoylation is related to toxicity effects and lipophilicity allows transport of the drug across the blood brain barrier. While all three aspects appear important for physiological activity, it was the correlation between therapeutic activity and alkylating ability of the nitrosoureas that was instrumental in initiating the work presented in this dissertation.

More recently[11] it was observed that 1-(2-chloroethyl)-1-nitrosourea 3 is a very active antitumor agent both *in vitro* and *in vivo* but does not generate an organic isocyanate upon aqueous decomposition. This observation supports the hypothesis that the antitumor activity of the nitrosoureas is due primarily to their ability to act as alkylating agents.

The effects of alkylating agents in biological systems at the molecular level are not well understood. Alkylating agents react with virtually every cell component and produce a number of biochemical results. The nitrosoureas

inhibit the synthesis of DNA, RNA and proteins *in vitro* and *in vivo*.[12] Research to elucidate the specific steps of this inhibition has shown that 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) 5 as well as 2-chloroethyl isocyanate inhibit nucleotidyltransferase activity to a larger extent than N-methyl-N-nitrosourea 2.[13] BCNU 5, CCNU 6, 2-chloroethyl isocyanate and cyclohexyl isocyanate inhibit the activity of *E. coli* DNA polymerase II but have no effect on DNA polymerase I.[14] DNA polymerase II is sensitive to thiol blocking agents,[15] and thus carbamoylation of the enzyme by isocyanates generated in the decomposition of nitrosoureas has been suggested[15] as the mechanism of this inhibition. Wheeler has concluded[15] that the biological effects of nitrosoureas are due to DNA damage by the alkylating portion of the molecule and inhibition of the repair process by the isocyanate generated.

DNA dependent RNA polymerase from Erlich ascites cells is inhibited by MNU 2 or N-propyl-N-nitrosourea,[16] while BCNU 5 has been shown to inhibit the transport of RNA from the nucleus.[17] Inhibition of protein synthesis has been interpreted as resulting from changes in polyribosomes after treatment with nitrosoureas.[18]

Alkylation may be generally defined according to the following equation:

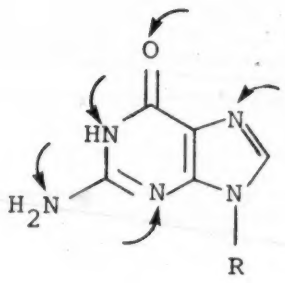
$$\text{Nuc.} + \text{R-L} \longrightarrow \text{R-Nuc.} + \text{L}^-$$

Nuc. is the nucleophile which is alkylated and R is an alkyl group attached to a leaving group L. There are essentially two courses for the alkylation to follow. At one extreme is the S_N2 process, in which Nuc. attacks R-L with concomitant loss of L^-. This reaction normally follows second order kinetics and is dependent on the concentration of both species. In contrast, the S_N1 process involves two steps, initial ionization of the alkylating agent to a carbonium ion R^+, followed by rapid reaction with the nucleophile. This reaction follows first order kinetics since the rate determining step, formation of the carbonium ion, is dependent only on the concentration of the alkylating agent.

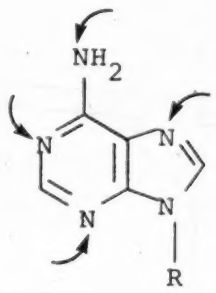
A number of factors may influence the course of a particular reaction. Where charged transition states or intermediates occur during the alkylation, polar solvents such as water will tend to lower activation energies and stabilize intermediates. Similar reactions occurring in nonpolar solvents
will be considerably slower. Neighboring groups can play an important role in assisting the displacement of L from R and producing stabilized intermediates which react as alkylating agents. Typical examples involving neighboring group participation include the sulfur and nitrogen mustards where chemically reactive three membered

aziridinium and sulfonium ions, respectively, are produced.[19]

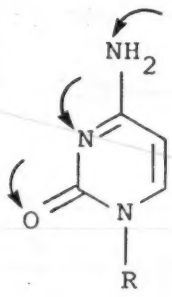
The products resulting from DNA alkylation depend upon the S_N1 or S_N2 character of the reaction and the reactivity of the particular site on the DNA macromolecule. The nucleophilic sites in DNA potentially resulting in base alkylation are shown below:



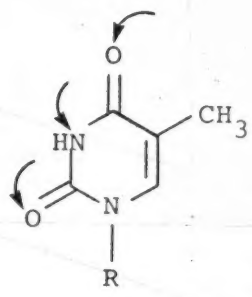
Guanosine



Adenosine



Cytidine



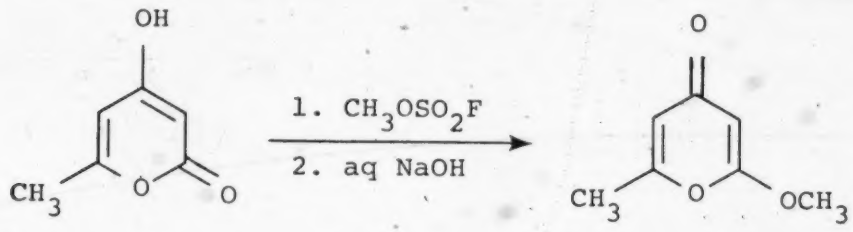
Thymidine

Typical S_N2 alkylating agents react with the 7 position of guanosine. 7-Alkylguanosine may account for 90% of the total base substitution.[20] A number of other sites including the 1,3 and 7 positions of adenosine and the 3 position of cytidine have also been shown to react with alkylating agents.[21]

The structures of the nucleosides shown above are the accepted major tautomeric forms observed in aqueous solution. A recent review by Beak[22] on the energies and alkylations of tautomeric heterocyclic compounds suggests that the extrapolation of tautomeric equilibrium constants from one molecular environment to another is unwise. The fact that tautomerization energies can be controlled by local molecular environment may result in the presence, in base paired hydrophobic areas of the DNA duplex, of "rare" tautomeric forms of purines and pyrimidines to a different degree than was thought possible on the basis of aqueous solution studies.

Beak[22] also suggests that if the factors which determine the ground state energy difference between tautomers, also control the relative transition-state energies for the first step of an alkylation, then the product formed will have the alkyl group attached to the heteroatom which does not bear the proton in the major tautomer. Such a result, which is more likely for cases involving a reactive alkylating agent and an early

transition state, have been reported for the reaction of methyl fluorosulfonate with various tautomeric heterocycles.[23] The conversion of 7 to 8 proceeds in 90% yield



7 8

upon reaction with this highly reactive methylating agent. Other procedures result in less than 25% conversion.

Recent research[24] suggests that a number of minor DNA alkylation products may be biologically more significant than alkylation at N-7 of guanosine. Ludlum[24] has reported that N-7 methylated poly G permits the incorporation of cytidine residues in the same manner as does poly G. Alkylation of the O-6 position of guanosine has been reported by Loveless.[25] This, in addition to cytidine N-3 alkylation, might result in significant mispairing and miscoding of bases. Lawley *et al.*[26,27] have described alkylation of the N-3 position of guanosine and the O-4 position of thymidine. Singer[28-30] has described the alkylation of the O-2 position of cytidine as well as nearly every potentially nucleophilic site of polyuridylic

acid including the 2'-O position of the ribose.

While alkylation of the internucleotide phosphate groups has been more difficult to establish, work by Ludlum[31] with poly A and by Freese and Rhaese[32] using dideoxynucleotides has shown indirectly that esterification of phosphates does occur significantly with ethylating agents. Bannon and Verly[33] have reported conclusive evidence for the formation of ethyl phosphotriesters in DNA and their stability under physiological conditions of pH 7.5 and 37°C. Phosphate alkylation may have a role in therapeutic activity since the phosphotriesters formed can proceed either chemically or enzymatically to DNA degradation in the form of single strand breaks.[21]

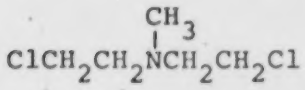
Alkylating agents which are bifunctional can of course undergo a second alkylation after initial attachment to the DNA. Bifunctional akylating agents have generally been observed[20] to be more lethal than their monofunctional counterparts. The formation of interstrand and/or intrastrand DNA cross-links between two guanine residues in the case of sulfur mustard have been observed by Brookes and Lawley.[34] Evidence for the existence of interstrand DNA cross-links for other bifunctional alkylating agents has been obtained using a number of techniques including reversible denaturation experiments,[35] spectrofluorometric assays[36] and inhibition of alkali-induced strand separation.[37]

Since the observation[21] during World War II that exposure to mustard gas [bis(2-chloroethyl)sulfide] 2 resulted in bone marrow suppression similar to that produced by radiation, interest in alkylating agents which are selective for fast proliferating cells has initiated a wide search for new and more selective drugs. The first such studies undertaken during World War II by a group at Yale University, involved the study of tris(2-chloroethyl)amine and its effect on diseases of the bone marrow. This study, later reported by Gilman,[38] was the first to establish the effectiveness of alkylating agents against certain malignancies as well as determining the two major disadvantages, (i) toxicity to the host and (ii) development of drug resistance by the tumor.

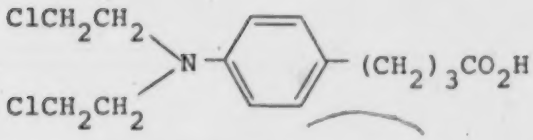
Clinically useful alkylating agents can be arranged in four basic categories, (i) sulfur and nitrogen mustards, (ii) aziridines and epoxides, (iii) methanesulfonates and (iv) nitrosoureas (Fig. 1). The last group of compounds, the nitrosoureas, are of very great practical and theoretical interest and are the subject of this dissertation.

The nitrosoureas have a certain specificity for neoplastic tissue, however, a number of toxicity factors including loss of hair, bone marrow depression and immunosuppression can be observed after treatment with these drugs. While the alkylating activity of the

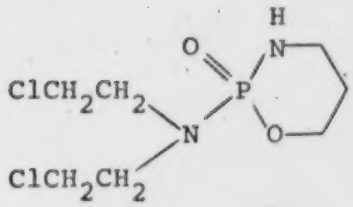
MUSTARDS



Nitrogen mustard

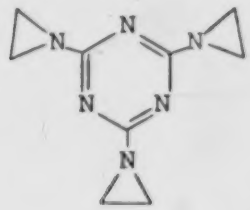


Chlorumbacil



Cyclophosphamide

AZIRIDINES AND EPOXIDES



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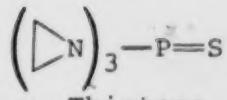
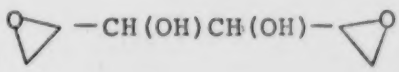
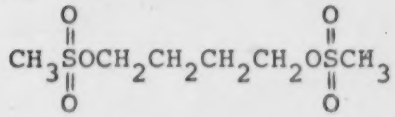


Thiotepa

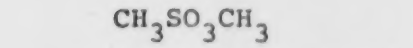


Dianhydrogalactitol

METHANESULFONATES

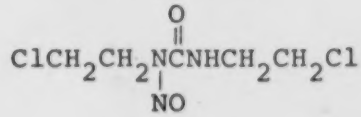


Bisulfan

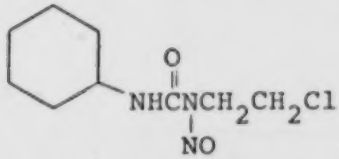


Methyl methanesulfonate

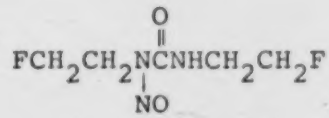
NITROSOUREAS



BCNU



CCNU



BFNU

Figure 1. Clinically useful alkylating agents (methyl methanesulfonate is listed for its theoretical interest only).

nitrosoureas appears to be related to therapeutic aspects,[5,8-10] the spectrum of activity is somewhat different than observed for other chemotherapeutic alkylating agents.[21] Tumors which have developed resistance to drugs such as the nitrogen mustards are sometimes sensitive to the nitrosoureas.[21]

The reasons for preferential cytotoxicity by nitrosoureas are not well understood. When the rates of macromolecular synthesis are used to measure the extents of cellular damage,[39-42] nitrosoureas appear to react with both host and neoplastic tissues, but, while the host tissues readily repair damage as measured by recovery of macromolecular synthesis[39-42] neoplastic tissues repair cellular damage only slowly.[39-42] While repair mechanisms are not fully understood, the selective effects of alkylating agents on neoplastic tissue may be related to such mechanisms rather than to an intrinsic difference in the alkylating reactions which occur in host and neoplastic cells.

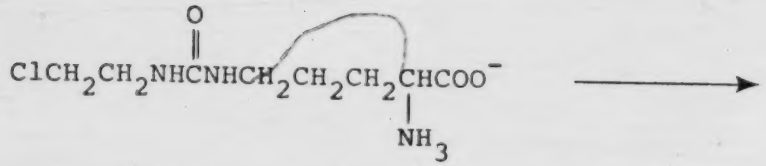
The original hypothesis[7] that N-methyl-N'-nitro-N-nitrosoguanidine 1 and N-methyl-N-nitrosourea 2 decompose under physiological conditions to produce diazomethane[7] has since been modified. Additional experiments[43-46] confirmed that alkylation of biological materials does occur by these compounds. Alkylation with N-methyl-N'-nitro-N-nitrosoguanidine 1, N-methyl-N-nitrosourea 2 or

N-ethyl-N-nitrosourea 10 labelled with ^{14}C and ^{3}H or ^{2}H in the methyl or ethyl group resulted in products with the same ratios of isotopes as in the parent compounds.[43-46] Since production of the diazoalkane intermediate requires proton loss from the methyl or ethyl group, this radioisotope work has led to the suggestion[15] that a diazohydroxide is the active alkylating agent. Additional work by Brundrett,[47] using BCNU-α-d_4 11, showed that upon aqueous decomposition, all of the 2-chloroethanol isolated contained two deuteriums.

$$ClCH_2CD_2N(NO)CONHCD_2CH_2Cl$$

11

The carbamoylating ability of the nitrosoureas has been observed by their reactions with lysine.[48-50] Addition of CCNU 4 to proteins followed by hydrolysis yielded N^6-cyclohexylcarbamoyl-lysine.[48,49] More recently it was shown[50] that carbamoylation of N^2 of lysine occurs more extensively than carbamoylation of N^6. Similar reactions with BCNU produced N^6-(2-chloroethylcarbamoyl)-lysine 12 which can cyclize even at room temperature to form oxazolinyl groups 13.[50]



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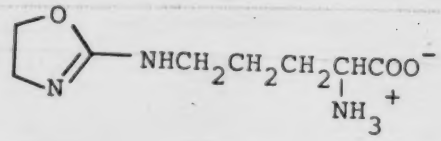

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In addition to carbamoylation and alkylation, nitrosoureas have been observed to be responsible for nucleic acid degradation,[51-53] but whether this degradation is a result of alkylation followed by enzymatic processes has not been determined. The present lack of understanding concerning the extent of DNA degradation and the processes so involved was additional impetus for the present study.

The primary objectives decided upon in an attempt to understand the chemical mechanisms by which the nitrosoureas exerted their antitumor effects were threefold. An investigation of the products of aqueous decomposition was undertaken to assist in determining the reactive intermediates involved. Since alkylating activity of the nitrosoureas has been observed to correlate with therapeutic effects, a study of DNA base alkylation was carried out using a sensitive ethidium bromide fluorescence assay. The mechanisms of DNA degradation also required additional investigation.

After a detailed examination of chemical mechanisms involved in the reactions of nitrosoureas with purified DNA, attempts were made to rationally design and synthesize new compounds which might exhibit superior reactivity with DNA. Extensive structure activity studies involving the nitrosoureas have been previously reported.[2,3,15] However, these investigations have generally resulted in modification of the carbamoylating portion of the molecule. The present study has involved two additional aspects: (i) the modification of nitrosoureas in an attempt to increase their alkylating ability and (ii) the design of compounds which might produce nitrosourea-like reactive intermediates. In addition to the *in vitro* assays outlined in subsequent chapters *in vivo* data was obtained for new compounds whenever possible.

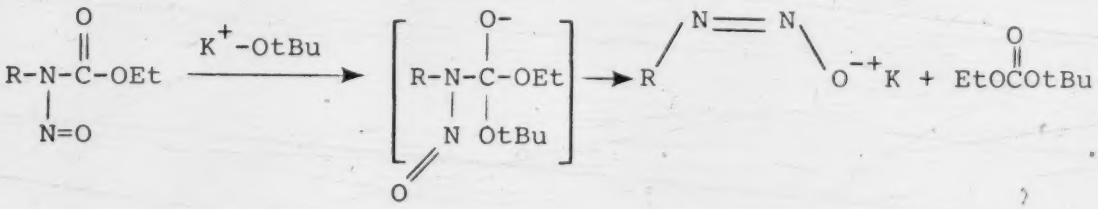
Rather than detail the extensive work which has been reported recently for the decomposition and chemical mode of action of the nitrosoureas at this time, a brief discussion of the relevant work as it applies to successive aspects of this study will introduce each of the subsequent chapters.

CHAPTER TWO

STUDIES RELATED TO THE SYNTHESIS AND DECOMPOSITION OF NITROSOUREAS

While there has been some disagreement as to whether base catalyzed decomposition of nitrosoureas such as N-methyl-N-nitrosoureas (MNU) 2 involves nucleophilic attack at the nitroso function[54] or the carbonyl,[55] or involves proton abstraction,[56] recent work[57] has indicated that the latter possibility is the more likely event. The diazotate 14, 15 (Fig. 2), produced under basic conditions, or the diazohydroxide 16, 17 (Fig. 2), existing in neutral solution can be of the *syn* 17 or *anti* 16 form (Fig. 2).[58] Rotation about the N-N bond is sufficiently restricted to prevent facile syn → anti isomerization.[59] *Syn*-diazotates can be independently prepared[60-62] by the action of potassium tertiary butoxide on the appropriate N-nitrosocarbamate (Scheme 1). They readily decompose[63]

Scheme 1



in aqueous media to form diazoalkanes 18 and/or carbonium ions 19 presumably by hydroxide ion elimination assisted by the anti positioning of the nitrogen lone pair (Fig. 2).

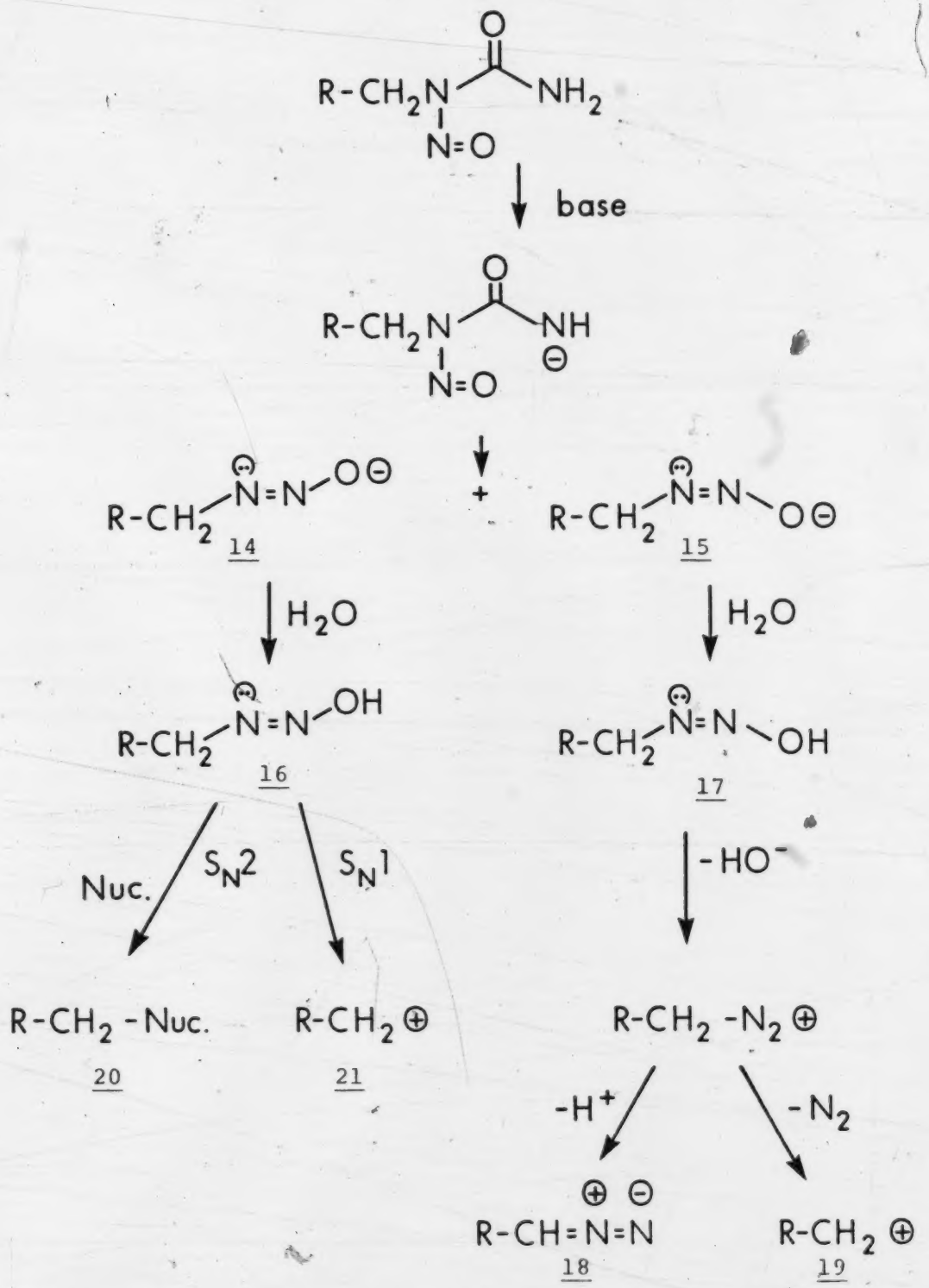
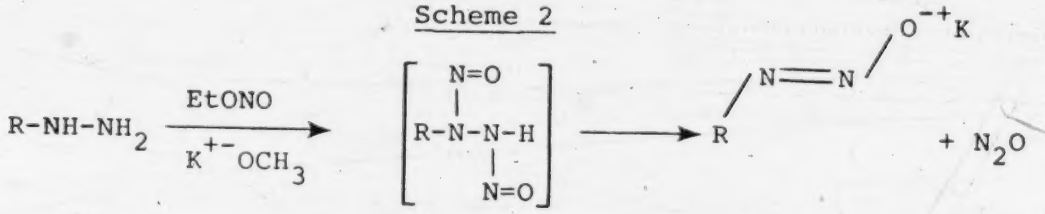


Figure 2. Base catalyzed decomposition of N-alkyl-N-nitrosoureas.

Anti-diazotates, prepared by nitrosation of monosubstituted hydrazines (Scheme 2)[60-62], are less reactive than the syn

Scheme 2



isomers and can be dissolved in cold water without reaction.[59] Heating induces diazoalkane and/or carbonium ion formation conceivably through anti → syn isomerization.[59] While the syn isomers readily undergo S_N1 type reactivity[63] it is possible that the anti analogues are predisposed to low activation S_N2 reactivity. Nucleophilic attack at the nitrogen bearing carbon would release the electron pair of the carbon-nitrogen bond to assist in an anti-elimination of hydroxide ion and produce the alkylated nucleophile 20 (Fig. 2). S_N1 reactivity can be expected of the anti isomer when it undergoes cleavage of the C-N bond with concomitant elimination of hydroxide ion resulting in a carbonium ion 21, a mechanism which has been suggested for the diazotization of primary amines.[64]

In 1967 Montgomery *et al.*[65] reported that the aqueous decomposition of BCNU 5 resulted primarily in formation of acetaldehyde with a small amount of 2-chloroethanol also present. Based on this result they suggested that a vinyl cation was the primary alkylating species generated

from BCNU 5 which produced acetaldehyde upon hydrolysis. Montgomery concluded[65] that BCNU 5 decomposes in an "abnormal" manner compared with MNU 2 and that rather than forming an alkyl diazohydroxide, loss of HCl initially produced a substituted 2-imino-N-nitrosooxazolidinone 22 (Fig. 3). Proton loss from this intermediate could then result in an isocyanate 23, a vinyl diazohydroxide 24 and/or a vinyl carbonium ion 25. Additional evidence for the vinyl alkylating species was obtained by the decomposition of BCNU 5 in a saturated sodium bromide solution.[66] GC mass spectral analysis of the decomposition mixture indicated that vinyl bromide, resulting from a vinyl alkylating species, was present. At this time they also reported that the ratio of 2-chloroethanol to acetaldehyde could vary significantly. In distilled water acetaldehyde predominated while in solutions buffered near physiological pH 2-chloroethanol was the major decomposition product. Conversely, 1,3-bis(2-fluoroethyl)-1-nitrosourea (BFNU) 26 was observed to decompose and produce almost exclusively 2-fluoroethanol, presumably due to the greater strength of the carbon-fluorine bond.

In 1974 research by Colvin *et al.*[67] indicated that the decomposition of BCNU 5 in a neutral aqueous buffer produced 2-chloroethanol and acetaldehyde in a 2.7:1 ratio (63% 2-chloroethanol, 23% acetaldehyde). Minor amounts of 1,2-dichloroethane and vinyl chloride were also

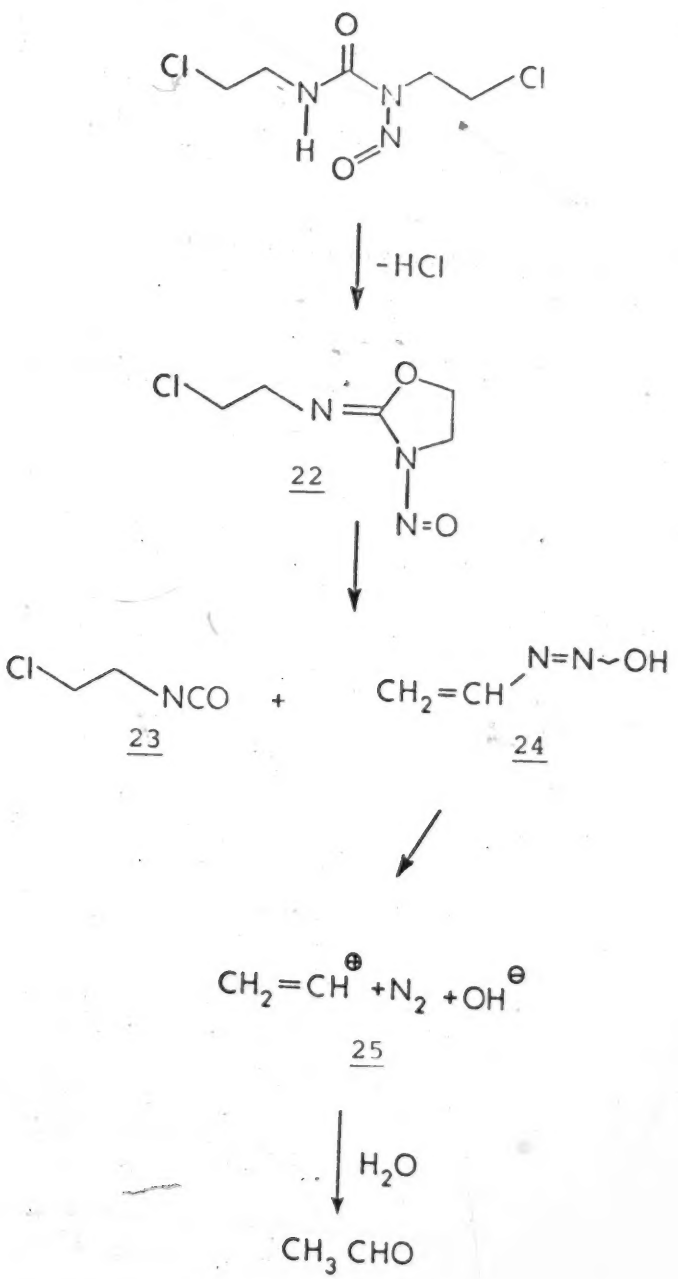


Figure 3. "Abnormal" decomposition suggested for BCNU 5.

identified. A second paper by Colvin *et al.*[11] reported that similar product ratios resulted from the decomposition of CNU 3 and CCNU 6 under comparable conditions. They suggested that 2-chloroethyl-nitrosoureas did in fact undergo "normal" decomposition compared with MNU 2, producing the 2-chloroethyl diazohydroxide 27 and/or 2-chloroethyl carbonium ion 28 (Fig. 4). Additionally, it was observed[11] that the treatment of chloroethylamine with nitrous acid produced 2-chloroethanol and acetaldehyde in a ratio similar to that previously observed for the aqueous decomposition of BCNU 5. Since the nitrosative deamination of amines is known to produce carbonium ions,[68] this product ratio led to the suggestion[11] that all of the decomposition products observed could be accounted for on the basis of a 2-chloroethyl carbonium ion 28 (Fig. 4). Research by Garrett and Goto[69] had indicated that N,N'-disubstituted nitrosoureas decomposed to produce carbonium ion-like species which were subject to rearrangements.

Investigation by Reed *et al.*[70] suggested that 2-chloroethanol was the major product from the decomposition of 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea 29 in aqueous buffer while 1-bromo-2-chloroethane resulted from the degradation of CCNU 6 in the presence of sodium bromide.

Brundrett *et al.*[47] investigated the decomposition of BCNU 5 which had been labelled with deuteriums on the

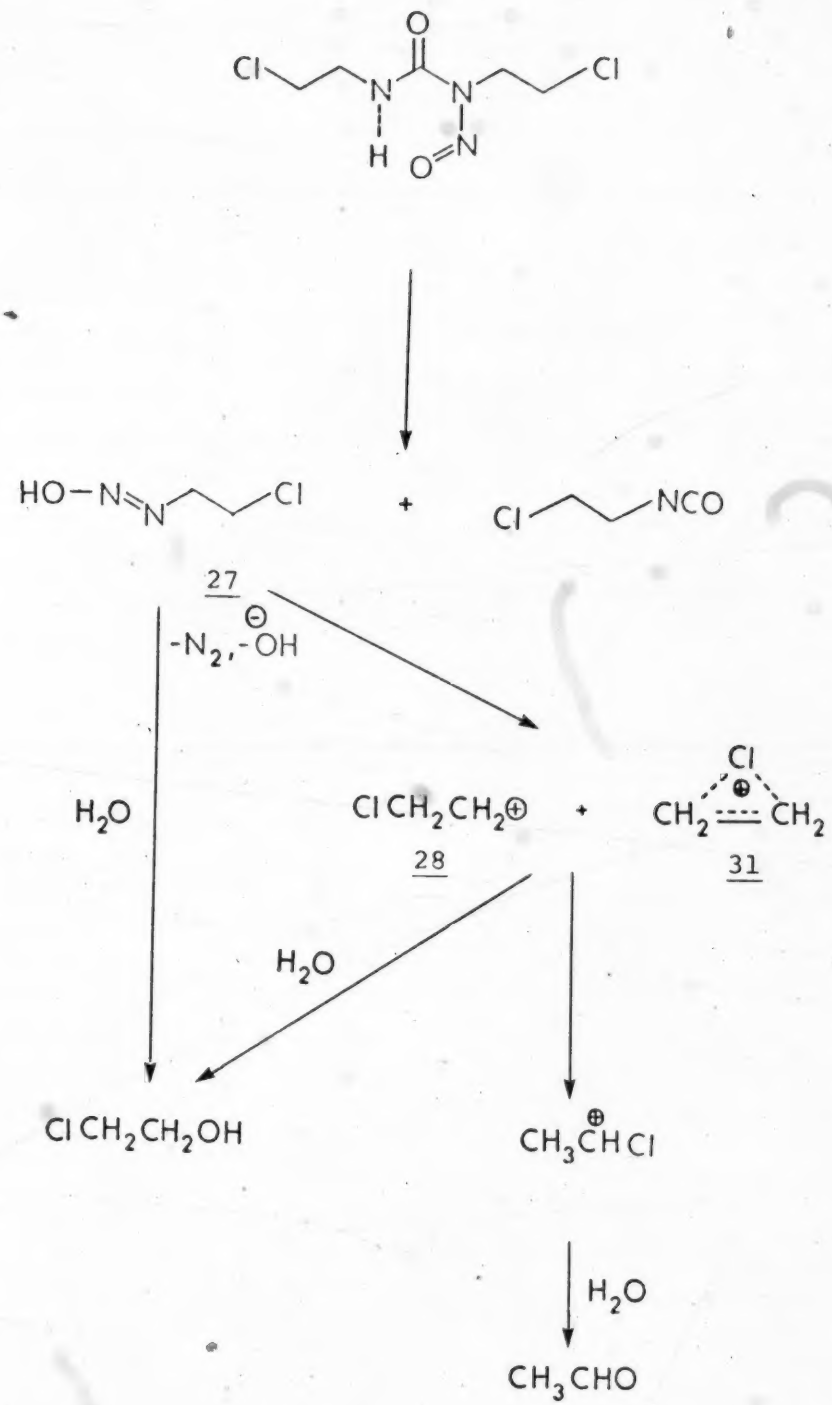


Figure 4. "Normal" decomposition suggested for BCNU 5.

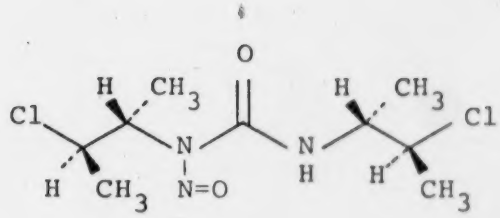
carbon atoms adjacent to the urea nitrogens (BCNU-α-d_4 11) or adjacent to the chlorine atoms (BCNU-β-d_4 30). The

$ClCH_2CD_2N(NO)CONHCD_2CH_2Cl$ $ClCD_2CH_2N(NO)CONHCH_2CD_2Cl$

11 30

observation that BCNU-α-d_4 11 produced acetaldehyde with no deuterium on the carbonyl carbon and that BCNU-β-d_4 30 produced acetaldehyde which contained a deuterium on the carbonyl carbon argued against the vinyl carbonium ion mechanism (see Fig. 3) proposed by Montgomery. Brundrett concluded[47] that rearrangement by hydride migration of the initial 2-chlorocarbonium ion followed by hydrolysis was the most likely mechanism to account for acetaldehyde production (Fig. 4). This same investigation reported, that in approximately 5% of the chloroethanol and 5% of the chloroethyl ether isolated (presumably produced by chloroethylation of some of the chloroethanol), both deuteriums had migrated to the adjacent carbon. This suggested that about 10% of the decomposition proceeds through a cyclic chloronium 31 ion (Fig. 4).

A second recent publication by Brundrett and Colvin[71] described the decomposition of 1,3-bis(*erythro*-3-chloro-2-butyl)-1-nitrosourea 32 and 1,3-bis(*threo*-3-chloro-2-butyl)-1-nitrosourea 33. Significantly more *threo*-alcohol



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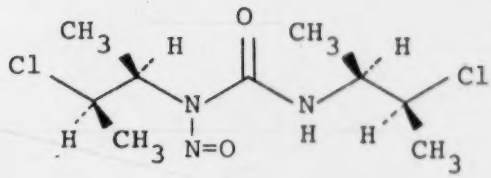

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was isolated from *erythro*-starting material as well as *erythro*-alcohol from *threo*-starting material. This implied that some attack by water occurred (ca. 1/3) by an S_N2 mechanism involving the diazohydroxide. The remaining alcohol resulted from S_N1 attack of the free carbonium ion or chloronium ion. Significant amounts of substituted vinyl chloride derivatives were also obtained, presumably through elimination reactions. Although the decomposition pathways for these compounds have been rigorously examined, a direct parallel with the decomposition pathways for BCNU 5 remains in doubt since both

carbon centers which potentially are involved in nucleophilic reactions are in this case secondary carbon atoms.

The present study was carried out in an attempt to clarify the chemistry involved in the decomposition of 2-haloethylnitrosoureas. Suitable modification of the 2-haloethylnitrosourea structure might increase the contribution of minor decomposition pathways (such as *via* the 2-imino-N-nitrosooxazolidinone) as well as confirm those previously suggested.[10,47,71] By determining the reactive intermediates involved in the aqueous decomposition of 2-haloethylnitrosoureas, subsequent investigations involving their reactions with DNA could begin.

Synthesis of Nitrosoureas

The nitrosoureas used in this study have been synthesized by reaction of the appropriate amine with the desired isocyanate followed by nitrosation. Structure modifications were usually carried out prior to the amine-isocyanate condensation as some reactions, particularly chlorinations with thionyl chloride, resulted in urea degradation (see the von Braun reaction[72]). Monosubstituted ureas were prepared by the reaction of an amine hydrochloride with potassium cyanate as shown in Table 1.

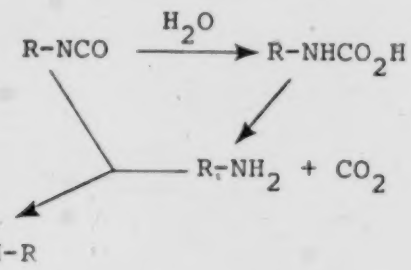
Table 1

$R\text{-}NH_2 \cdot HCl + KOCN \rightarrow R\text{-}NHCONH_2 + KCl$

R	Compound
CH_3-	N-methylurea 34
CH_3CH_2-	N-ethylurea 35
$ClCH_2CH_2$-	2-chloroethylurea 36
$ClCH_2CH_2CH_2$-	3-chloropropylurea 37
$ClCH_2CH_2CH_2CH_2$-	4-chlorobutylurea 38
$ClCH_2CH_2CH_2CH_2CH_2$-	5-chloropentylurea 39

Symmetrical disubstituted ureas in some cases were prepared by addition of the appropriate isocyanate to water containing triethylamine. Initial hydrolysis of the isocyanate to the carbamic acid followed by decarboxylation produced *in situ* an amine. Reaction of the amine with remaining isocyanate resulted in the symmetric ureas shown in Table 2.

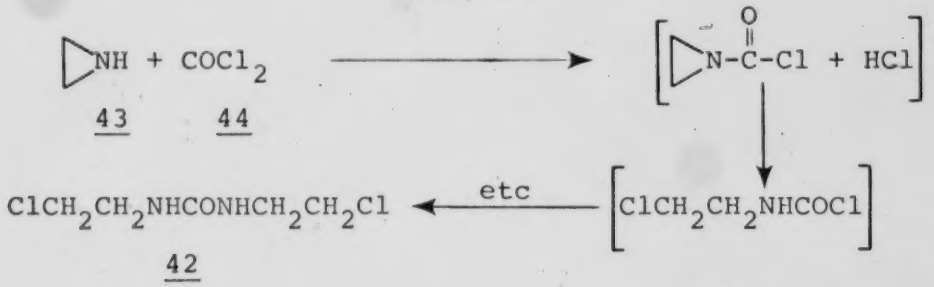
Table 2



R	Compound
$ClCH(CH_3)CH_2$-	1,3-Bis(2-chloropropyl)-1-nitrosourea 40
$ClCH_2CH(CH_3)$-	1,3-Bis[1-(chloromethyl)ethyl]-1-nitrosourea 41

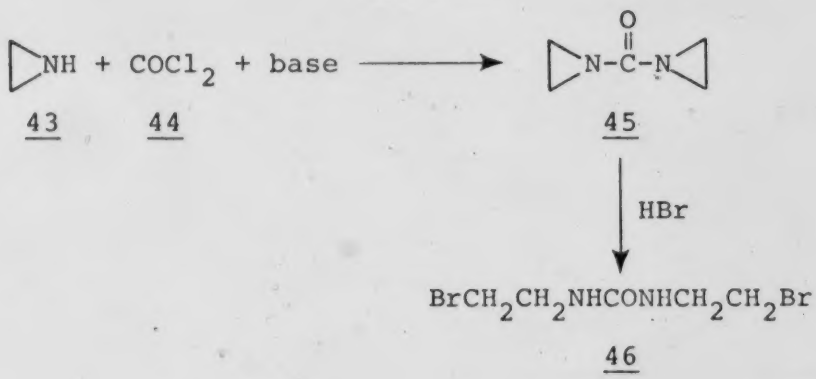
1,3-Bis(2-chloroethyl)urea 42 was prepared[3] by the slow addition of aziridine 43 to phosgene 44 as shown in Scheme 3. Nucleophilic attack by aziridine on the carbonyl

Scheme 3



releases hydrogen chloride which opens the aziridine ring. Addition of aziridine 43 to phosgene 44 in the presence of a base produces carbonyl-1,1-bisaziridine 45 which can then be treated[3] with aqueous hydrobromic acid to produce 1,3-bis(2-bromoethyl)urea 46 (Scheme 4). 1,3-Bis(2-

Scheme 4



iodoethyl)urea 47 results when 1,3-bis(2-chloroethyl)urea 42 is treated with sodium iodide in refluxing acetone.

Unsymmetrical disubstituted ureas can be prepared, in principle, by two pathways. Addition of 2-chloroethylamine to phenyl isocyanate or addition of aniline to 2-chloroethyl isocyanate both result in 1-(2-chloroethyl)-3-phenylurea 48. Compounds prepared by this route are shown in Table 3.

Table 3

$$R\text{-}NCO + R'\text{-}NH_2 \rightarrow R\text{-}NHCONH\text{-}R'$$

R	R'	urea
$ClCH_2CH_2$-	C_6H_5-	1-(2-chloroethyl)-3-phenylurea 48
C_6H_5-	$ClCH_2CH_2$-	1-(2-chloroethyl)-3-phenylurea 48
C_6H_{11}-	$ClCH_2CH_2$-	1-(2-chloroethyl)-3-cyclohexylurea 49
C_6H_{11}-	FCH_2CH_2-	3-cyclohexyl-1-(2-fluoroethyl)urea 50
C_6H_{11}-	$BrCH_2CH_2$-	1-(2-bromoethyl)-3-cyclohexylurea 51
C_6H_{11}-	$HOCH_2CH_2$-	3-cyclohexyl-1-(2-hydroxyethyl)urea 52
C_6H_{11}	$CH_3OCH_2CH_2$-	3-cyclohexyl-1-(2-methoxyethyl)urea 53
$ClCH_2CH_2$-	p-$CH_3OC_6H_5$-	1-(2-chloroethyl)-3-p-methoxyphenylurea 54
$ClCH_2CH_2$-	p-$NO_2C_6H_5$-	1-(2-chloroethyl)-3-p-nitrophenylurea 55
$ClCH_2CH_2$-	$(CH_3)_2$-	1-(2-chloroethyl)-3,3-dimethylurea 56

Triethylamine can be used to conserve an expensive amine by avoiding prior isolation of the free base. Thus, 1-cyclohexyl-3-(2-fluoroethyl)urea 50 was prepared from

cyclohexyl isocyanate, 2-fluoroethylamine hydrochloride and excess triethyl amine.

Nitrosation of the ureas was always the final synthetic step as the products are unstable to heat as well as to basic conditions and, therefore, subsequent synthetic steps were not feasible. Three methods of nitrosation were employed:

(i) Aqueous nitrosation using sodium nitrite in dilute hydrochloric or sulfuric acid was most valuable for monosubstituted and symmetrical disubstituted ureas providing they had some water solubility as shown in Table 4.

Table 4

$$RNHCONHR' \xrightarrow[HCl \text{ or } H_2SO_4]{NaNO_2} RN(NO)CONHR'$$

R	R'	Compound
CH_3-	-H	N-methyl-N-nitrosourea 2
CH_3CH_2-	-H	N-ethyl-N-nitrosourea 10
$ClCH_2CH_2$-	-H	1-(2-chloroethyl)-1-nitrosourea 3
$ClCH_2CH_2$-	$-CH_2CH_2Cl$	1,3-bis(2-chloroethyl)-1-nitrosourea 5

(ii) Nitrosation in 98% formic acid using solid sodium nitrite added portionwise was most effective for compounds which were not water soluble, as well as unsymmetrical disubstituted ureas (Table 5). Under these conditions,

Table 5

$$RNHCONHR' \xrightarrow[HCO_2H]{NaNO_2} RN(NO)CONHR'$$

R	R'	Compound
$ClCH_2CH_2CH_2$	-H	1-(3-chloropropyl)-1-nitrosourea 57
$ClCH_2CH_2CH_2CH_2$-	-H	1-(4-chlorobutyl)-1-nitrosourea 58
$ClCH_2CH_2CH_2CH_2CH_2$-	-H	1-(5-chloropentyl)-1-nitrosourea 59
$ClCH(CH_3)CH_2$-	$-CH_2CH(CH_3)Cl$	1,3-bis(2-chloropropyl)-1-nitrosourea 60
$ClCH_2CH(CH_3)$	$-CH(CH_3)CH_2Cl$	1,3-bis[1-(chloromethyl)ethyl]-1-nitrosourea 61
$BrCH_2CH_2$-	$-CH_2CH_2Br$	1,3-bis(2-bromoethyl)-1-nitrosourea 62
ICH_2CH_2-	$-CH_2CH_2I$	1,3-bis(2-iodoethyl)-1-nitrosourea 63
FCH_2CH_2-	$-C_6H_{11}$	3-cyclohexyl-1-(2-fluoroethyl)-1-nitrosourea 64
$ClCH_2CH_2$-	$-C_6H_{11}$	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea 6
$BrCH_2CH_2$-	$-C_6H_{11}$	1-(2-bromoethyl)-3-cyclohexyl-1-nitrosourea 65
$HOCH_2CH_2$-	$-C_6H_{11}$	3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea 66
$CH_3OCH_2CH_2$-	$-C_6H_{11}$	3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea 67
$ClCH_2CH_2$-	$-p-C_6H_5OCH_3$	1-(2-chloroethyl)-3-p-methoxyphenyl-1-nitrosourea 68
$ClCH_2CH_2$-	$-p-C_6H_5NO_2$	1-(2-chloroethyl)-3-pnitrophenyl-1-nitrosourea 69
$ClCH_2CH_2$	$-(CH_3)_2$	1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea 70

unsymmetrical disubstituted ureas were nitrosated at the less hindered amidic nitrogen as first observed by Montgomery.[3] Proton magnetic resonance analysis of the products confirmed this initial observation for the nitrosoureas prepared in this study. Mixtures of the isomeric nitrosation products can be observed when the formic acid contains as little as 5-10% water.

Sodium nitrite in formic acid most probably results in formyl nitrite 71, a species reportedly observed spectroscopically.[73] Montgomery suggests[3] that the transfer of the nitroso group in 98% formic acid occurs through the cyclic intermediate 72 shown in Figure 5. The use of a cyclohexyl or similar bulky R group attached to one of the urea nitrogens results in exclusive nitrosation at the 2-chloroethyl amidic position. The isomeric composition appears to depend on the relative stabilities of the two possible cyclic intermediates.

(iii) Dinitrogen tetraoxide (N_2O_4) in dry ether resulted in good yields of N-nitroso derivatives. It does not result in the regioselectivity of the previously discussed method but is especially valuable in cases where the product exhibits water solubility and remains in the aqueous nitrosating medium.

Decomposition of the Nitrosoureas

Figure 6 outlines detailed mechanistic steps of the decomposition pathways as suggested by research outlined

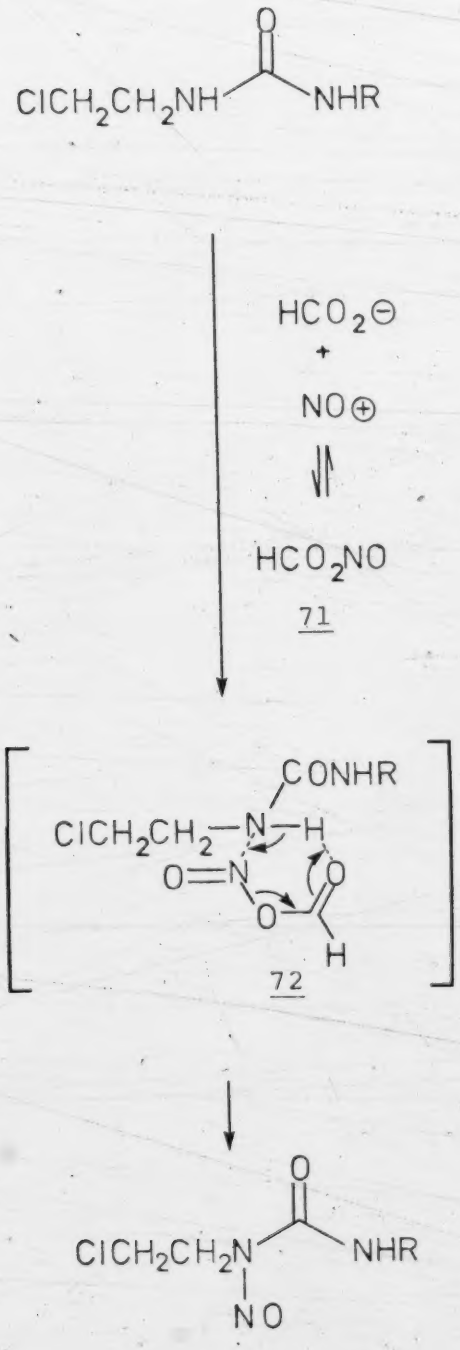


Figure 5. Selective N-nitrosation at the less sterically hindered amidic nitrogen.

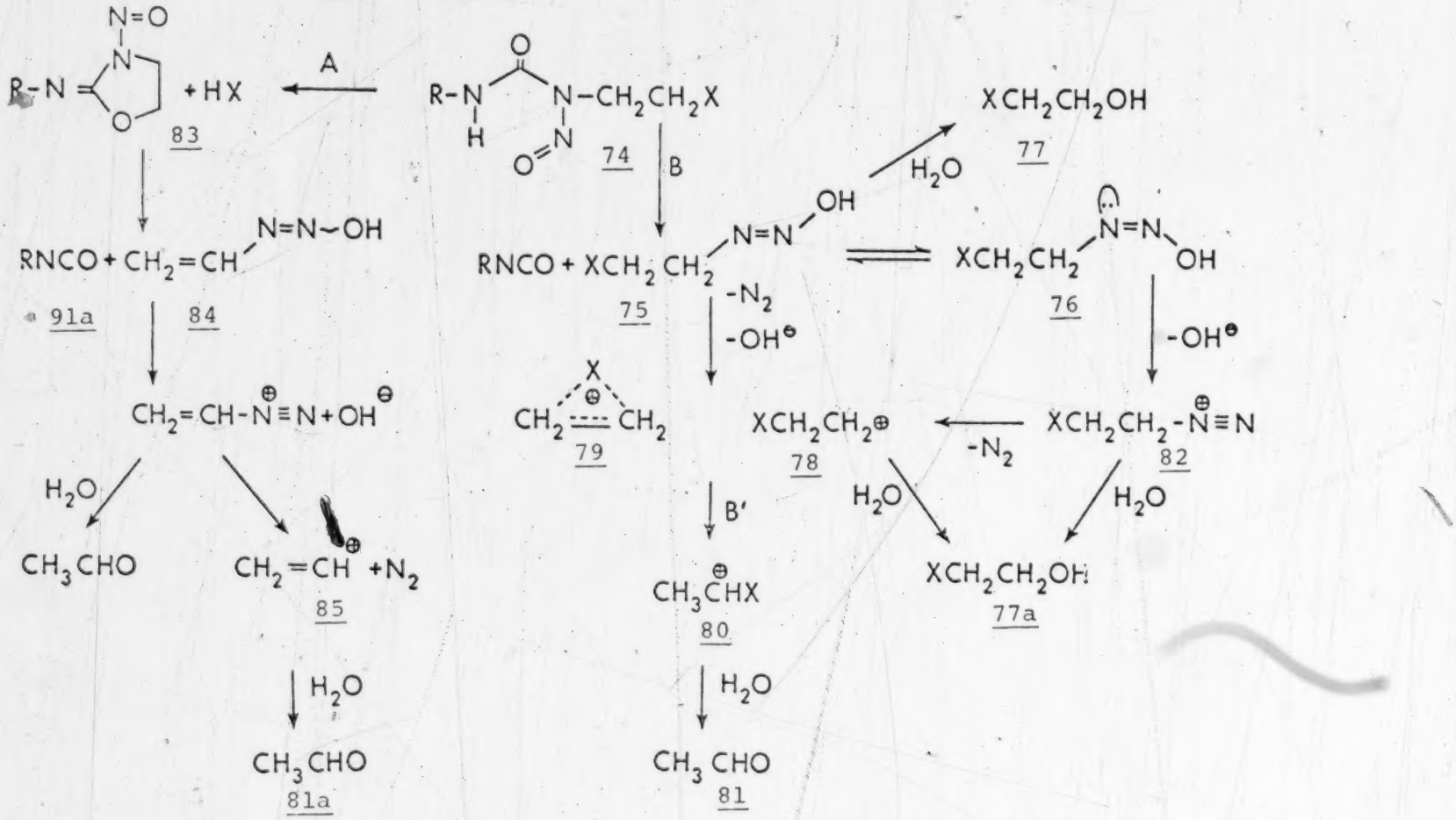


Figure 6. Suggested decomposition pathways for 2-haloethylnitrosoureas.

in the introduction. When the 2-haloethylnitrosourea 74 decomposes in a "normal" manner (Pathway B) the *anti*-diazohydroxide 75 and/or the *syn*-diazohydroxide 76 are produced. S_N2 reactivity by the anti isomer results in haloethanol 77 while S_N1 reactivity produces the 2-haloethyl carbonium ion 78 or cyclic haloethonium ion 79. These intermediates may undergo hydride transfer (Pathway B') to form the halocarbonium ion 80, which upon hydrolysis produces acetaldehyde 81. Olah has reported[74] that in superacid media the cyclic haloethonium ion 79 and halocarbonium ion 80 do not interconvert. Hydrolysis of the 2-haloethyl carbonium ion 78 or cyclic haloethonium ion 79 produces 2-haloethanol 77a. S_N1 reactivity would be expected from the *syn*-diazohydroxide 76 producing the alkyl diazonium ion 82 which may react directly with water or, lose nitrogen to produce 78 and 79.

"Abnormal" decomposition (Pathway A) results in the N-substituted 2-imino-N'-nitrosooxazolidinone 83 which decomposes to form the diazohydroxides 84. S_N2 reactivity at an sp^2 hybridized carbon is unlikely so reaction of the vinyl diazohydroxides 84 is most probably by S_N1 to form the vinyl carbonium ion 85, which results in acetaldehyde 81a.

Studies Related to the Decomposition Rates of Nitrosoureas

The first aspect of the investigation of the decomposition of nitrosoureas involved the determination of their

stabilities in aqueous pH 7.1 buffered solution at 37°C. Under these conditions nitrosoureas decompose to produce their reactive intermediates without enzymatic activation.[15] While Wheeler *et al.*[75,76] have used the uv absorbance of the nitroso function to monitor decomposition rates in 5% ethanol/water buffered to pH 7.4, other methods have been less direct. Loo and Dion[77] developed a colorimetric procedure based on the release of nitrous acid and Montgomery *et al.*[6] measured the rates of nitrogen and carbon dioxide evolution during decomposition. The latter method involves analysis after a series of steps and measures overall rates of decomposition to form final products. Polarographic analysis employing the electrochemically active nitroso group proved to be a convenient and sensitive method for determining directly the rate of the first step of the decomposition of the nitrosoureas.

All of the nitrosoureas studied showed two well-defined, polarographic waves independent of pH, and the polarographic parameters $E_{1/2}$ and i_{lim} were easily measured. These waves in neutral solution correspond to the reversible reduction of the N-nitroso group to the hydroxyamino group[78] followed by reduction to the amino group, both processes requiring two electrons. The two waves are often but not always of equal height. In all cases these waves decreased with time following the aqueous decomposition of the nitrosoureas (Table 6). In no cases were

Table 6

Polarographic Behavior of Nitrosoureas

R-N(NO)CONHR'

#	R	R'	$E_{1/2}$, 1	$E_{1/2}$, 2	$k(\times 10^{-3})$ min^{-1}	$t_{1/2}$ (min)
2	CH_3-	-H	-0.884	-1.041	14.8	7±2
10	CH_3CH_2-	-H	-0.955	-1.155	43.3	16±1
3	$ClCH_2CH_2$-	-H	-0.752	-1.010	88	8±4
57	$Cl(CH_2)_2CH_2$-	-H	-0.785	-1.025	116	6±2
58	$Cl(CH_2)_3CH_2$-	-H	-0.982	-	128	5±0.5
59	$Cl(CH_2)_4CH_2$-	-H	-0.980	-	110	5±0.5
26	FCH_2CH_2-	$-CH_2CH_2F$	-0.890	-1.117	8.9	78±2
5	$ClCH_2CH_2$-	$-CH_2CH_2Cl$	-0.777	-1.110	8.8	79±5
62	$BrCH_2CH_2$-	$-CH_2CH_2Br$	-0.705	-1.095	16.9	52±3
63	ICH_2CH_2-	$-CH_2CH_2I$*	-0.775	-1.035	9.6	58±3
64	cyclo-C_6H_{11}-	$-CH_2CH_2F$	-0.724	-1.050	9.5	73±2
6	cyclo-C_6H_{11}-	$-CH_2CH_2Cl$*	-0.853	-1.168	10.0	59±1

continued.....

Table 6 (continued)

65	cyclo-C_6H_{11}-	$-CH_2CH_2Br$*	-0.845	-1.075	36.5	19±1
66	cyclo-C_6H_{11}-	$-CH_2CH_2OH$	-0.771	-1.034	3.7	186±6
67	cyclo-C_6H_{11}-	$-CH_2CH_2OCH_3$	-0.823	-1.125	0.5	1445±30
60	$ClCH(CH_3)CH_2$-	$-CH_2CH(CH_3)Cl$	-0.835	-1.135	9.3	74
61	$ClCH_2CH(CH_3)$-	$-CH(CH_3)CH_2Cl$	-0.865	-1.015	32.1	22
86	chlorozotocin		-0.770	-1.112	17.8	39±1
87	streptozotocin		-0.960	-1.140	16.9	41±1
88	GANU		-0.755	-1.035	70.7	10±1
89	N-nitrosooxazolidinone		-0.705	-1.145	16.9	41±2
70	$(CH_3)_2NCON(NO)CH_2CH_2Cl$		-0.870	-1.115	0.25	>2800

* = 5% CH_3CN.

any waves from reducible decomposition products observed. This implies that the decomposition products are transient and/or electrochemically inactive in accord with the suggested primary decomposition pathways (Fig. 6).

Plots of the logarithm of the diffusion-limited current against time were always linear for both waves permitting determination of the rate constants for the decomposition at pH 7.10. Values of the rate constants were determined separately for each wave. In nearly all cases these two calculated rate constants were identical within experimental error and the reported values are the mean of at least two measurements; the error cited is the author's estimate and varies with number of kinetic runs, definition of the polarographic waves, and linearity of the logarithmic plots. The reported rate constant values are calculated from half-life values measured over at least one half-life except for very slow decompositions. The rates and potentials in the partially nonaqueous cases are not strictly comparable with those in aqueous solution. For example, BCNU in aqueous solution had a half-life of 79 min, however, in 5% acetonitrile the half-life dropped to 52 min (Table 7). A similar change is observed for BBNU.

The rates of the first step of the decompositions of the nitrosoureas measured electrochemically (Table 6) are not entirely in agreement with previous studies.[75,76]

Table 7

Solvent Effects on the Decomposition Rates of Nitrosoureas

RH(NO)CONHR'

#	R	R'	solvent	$t_{1/2}$(min)
5	$ClCH_2CH_2$-	$-CH_2CH_2Cl$	H_2O pH 7.1	79
5	$ClCH_2CH_2$-	$-CH_2CH_2Cl$	5% acetone	72
5	$ClCH_2CH_2$-	$-CH_2CH_2Cl$	5% ethanol	69
5	$ClCH_2CH_2$-	$-CH_2CH_2Cl$	5% acetonitrile	52
62	$BrCH_2CH_2$-	$-CH_2CH_2Br$	H_2O pH 7.1	52
62	$BrCH_2CH_2$-	$-CH_2CH_2Br$	5% acetonitrile	36

However, it is evident from Table 7 that the decomposition rates are highly dependent on the solvent system. Therefore, it is not unexpected that the rates measured in the present study differ from those of Wheeler in which case the compounds were examined in 5% ethanol with the compound initially dissolved in acetone.[75,76]

It may be observed from Table 8 that, as anticipated, the rate of decomposition of BCNU 5 increases progressively with increasing pH in the range 4.4 to 8.0.

A free $-NH_2$ group in the urea structure as in CNU 3, MNU 2, ENU 10, 1-(3-chloropropyl)-1-nitrosourea 57, 1-(4-chlorobutyl)-1-nitrosourea 58, and 1-(5-chloropentyl)-1-nitrosourea 59 considerably accelerates the rate of

Table 8

pH Effects Upon Decomposition Rates

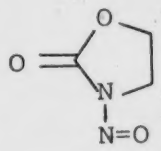
#	Compound	temp.	pH	$t_{1/2}$ min
5	BCNU	22°	4.4	3890±90
5	BCNU	22°	7.0	734±70
5	BCNU	22°	8.0	481±15

decomposition relative to BCNU 5 or CCNU 6 (Table 1). Lack of an N-H proton as in 1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea 70 severely inhibits decomposition in agreement with previous work[11] (Table 6). These results suggest that loss of the N-H proton is the first step of the decomposition of 2-haloethylnitrosoureas in accord with recent results of Hecht and Kozarich[56,57] involving the decomposition of N-methyl-N-nitrosourea 2.

It is evident from Table 6 that compounds which have identical structures, except for the halogen substituent, do not always have similar decomposition rates. While BFNU 26 and BCNU 5 have comparable half-lives, BBNU 62 and BINU 63 decompose significantly faster (BINU 63 is measured in 5% acetonitrile and, therefore is not strictly comparable). It can also be observed that in the series of compounds which contain a cyclohexyl group and a 2-substituted ethyl nitrosourea (62, 5, 26, 66, 67), the half-lives vary considerably (Table 1).

For the substituents -Br, -Cl, -F, -OH and $-OCH_3$ the respective half-lives are 19, 69, 73, 186 and 1445 min (Table 6). As the leaving ability of the substituent decreases the half-life increases (again the -Br and -Cl analogues are not strictly comparable). This implies that loss of the substituent after initial proton abstraction (or transfer) is a significant decomposition pathway for some nitrosoureas.

Since a 2-imino-N -nitrosooxazolidinone 83, (Fig. 6) intermediate first suggested by Montogomery[65] is in agreement with the two previous observations (proton abstraction followed by loss of the halogen), the electrochemistry of N-nitrosooxazolidinone 89[79] was investigated.



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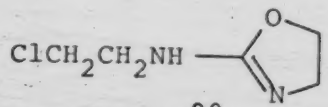
The polarographic data for this compound are listed in Table 6. Its rate of hydrolysis under the same conditions is considerably faster than that observed for fluoro- and chloroethylnitrosoureas but comparable to the bromo- and iodo- derivatives. The two half-wave potentials are so close to those observed for the 2-haloethylnitrosoureas

that it cannot be distinguished from them in dilute solutions. The observation by Montgomery[6] that 2-(2-chloroethylamino)-2-oxazoline 90 is isolated after aqueous buffered decomposition of BCNU 5 as well as the observation[40] that carbamoylation of lysine at the N^6 or N^2 position with 2-chloroethylisocyanate results in cylization to form oxazolinyl groups (13 Chapter I) indicates that cyclizations similar to that suggested by Montgomery are



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known. However, whether a 2-imino-N-nitrosooxazolidine 83 (Fig. 6) is an intermediate in the decomposition of nitrosoureas cannot be determined from the electrochemical data presented.

From rate data at different temperatures (separate study, Table 9) the Arrhenius parameters were derived for BFNU 26, BCNU 5, BBNU 62 in aqueous pH 7.1 solutions as follows: log A, -20.1 ± 1.4, -21.6 ± 0.7, -22.3 ± 1.6, Ea, 24.4 ± 2.0, 26.5 ± 1.0, 27.2 ± 2.3 kcal/mole. Despite all efforts no results could be obtained for BINU 63 in aqueous solution, and so BBNU 62 and BINU 63 were both examined in 4.8% acetonitrile with the following results: log A, 18.9 ± 1.6, 19.9 ± 1.0; Ea 24.0 ± 1.5, 24.8 ± 1.5 kcal/mole. On the basis of se results we estimate the values for log A and Ea for I J 63 in

Table 9

Temperature Dependence of Nitrosourea Hydrolysis Reaction

Compound	Temperature (°C)	half-life (min)	log k (k, sec^{-1})
BFNU 26	28	220	-2.436
BFNU 26	37	76	-2.898
BFNU 26	41	38	-3.202
BFNU 26	47	20	-3.468
BCNU 5	28	288	-2.318
BCNU 5	37	84	-2.852
BCNU 5	41	49	-3.085
BCNU 5	47	20	-3.468
BBNU 62	28	161	-2.571
BBNU 62	37	52	-3.063
BBNU 62	41	24	-3.403
BBNU 62	47	11	-3.729
BBNU* 62	28	103	-2.765
BBNU* 62	37	36	-2.317
BBNU* 62	41	27	-3.353
BBNU* 62	47	11	-3.745
BINU* 63	28	182	-2.517
BINU* 63	37	58	-3.012
BINU* 63	41	39	-3.189
BINU* 63	47	16	-3.566

*In 5% CH_3CN (v/v); otherwise aqueous; pH 7.1.

aqueous solution as: log A, -23.3 ± 3.0; Ea, 28.0 ± 3.0 kcal/mole. The plots of the logarithm of the diffusion current against time from which the rate data were derived were in all cases linear over at least one half-life. The Arrhenius plots were also in all five cases linear and the error limits given are the standard deviations. The values of E* obtained in this study are within experimental error of those obtained for similar compounds under similar but not identical conditions in the spectrometric study of Garrett and Goto.[69]

Studies Related to the Products Resulting from the Decomposition of 1,3-Bis(2-haloethyl)-1-nitrosoureas

The 2-haloethylnitrosoureas are considered to undergo decomposition by two major pathways (Fig. 6) yielding as major products 2-haloethanol 77, 77a, acetaldehyde 81, 81a and an isocyanate 91, 91a. While the isocyanate 91, 91a is produced in either pathway A or pathway B, 2-haloethanol 77, 77a is only produced through pathway B. Acetaldehyde 81, 81a can be produced through either mode of decomposition, however, Brundrett's work[47] has indicated that the contribution of the cyclic chloronium ion 79, and presumably acetaldehyde 81 *via* pathway B, in the decomposition of BCNU 5, is only 10% of the total as measured by deuterium scrambling in the products isolated. Therefore a relative increase in acetaldehyde production in the

decomposition of a series of related nitrosoureas might indicate a significant contribution by pathway A *via* the 2-imino-N-nitrosooxazolidinone 83 (Fig. 6). A comparison of the product ratios after decomposition was made for four nitrosoureas; BFNU 26, BCNU 5, BBNU 62 and BINU 63 (Table 10). The four derivatives were suspended in a

Table 10

Decomposition of 1,3-Bis(2-haloethyl)-1-nitrosoureas

$XCH_2CH_2N(NO)CONHCH_2CH_2X$

X	% CH_3CHO	% XCH_2CH_2OH
F	18	80
Cl	25	61
Br	39	14
I	66	0

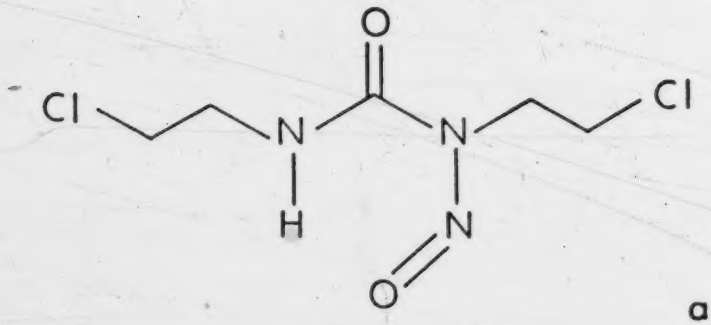
pH 7.2 buffer and incubated at 37°C in a sealed glass vial for 24 h. Product analysis of the decomposition mixture was made, using gas-liquid chromatography immediately upon opening the vials. The percentages of 2-haloethanol and acetaldehyde are listed in Table 10. The products obtained for BCNU 5 and BFNU 26 are in agreement with a previous investigation.[11]

It is evident from Table 10 that the percentage of acetaldehyde produced in the decomposition of 1,3-bis(2-haloethyl)-1-nitrosoureas increases in the series fluorine,

chlorine, bromine and iodine. This may result, in the case of BBNU 62 and BINU 63, from decomposition *via* pathway A (Fig. 6) where the superior leaving ability of bromide and iodide relative to chloride and fluoride may facilitate intramolecular nucleophilic displacement to produce the 2-imino-N-nitrosooxazolidinone 83. However, the polarizability and thus the stability of the halocarbonium ion 80 (Fig. 6) also increases in the series fluorine, chlorine, bromine and iodine. Therefore, pathway B' (Fig. 6) can also be expected in a greater proportion for BBNU 62 and BINU 63 than BFNU 26 and BCNU 5. Clearly additional experiments were required to determine the decomposition pathways involved with the 2-haloethylnitrosoureas.

Studies Related to the Decomposition of Methyl Substituted BCNU Derivatives

Steric effects as they relate to the decomposition of 2-chloroethylnitrosoureas were investigated using three appropriately substituted derivatives: 1,3-bis(2-chloroethyl)-1-nitrosourea 5 (BCNU), 1,3-bis(2-chloropropyl)-1-nitrosourea 60 (BCNU-β-Me) and 1,3-bis[1-(chloromethyl)-ethyl]-1-nitrosourea 61 (BCNU-α-Me) (Fig. 7). While acetaldehyde results from the decomposition of BCNU 5 either *via* pathway A (Fig. 8), or *via* pathway B following a hydride shift (Fig. 9), the same is not true for the



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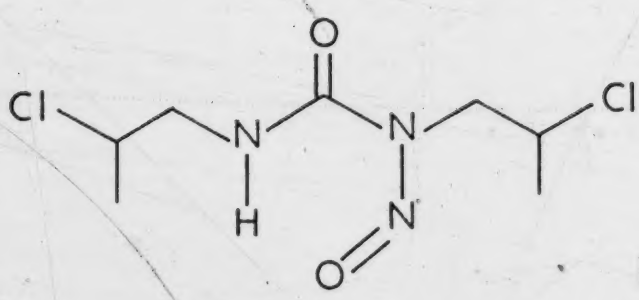

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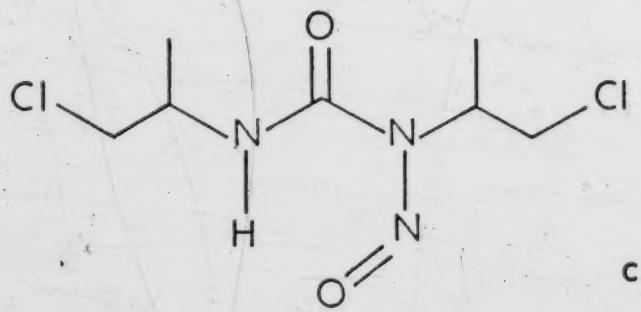


c.

Figure 7. (a) 1,3-bis(2-chloroethyl)-1-nitrosourea 5 BCNU.

(b) 1,3-bis(2-chloropropyl)-1-nitrosourea 60 (BCNU-β-Me).

(c) 1,3-bis[1-(chloromethyl)ethyl]-1-nitrosourea 61 (BCNU-α-Me).

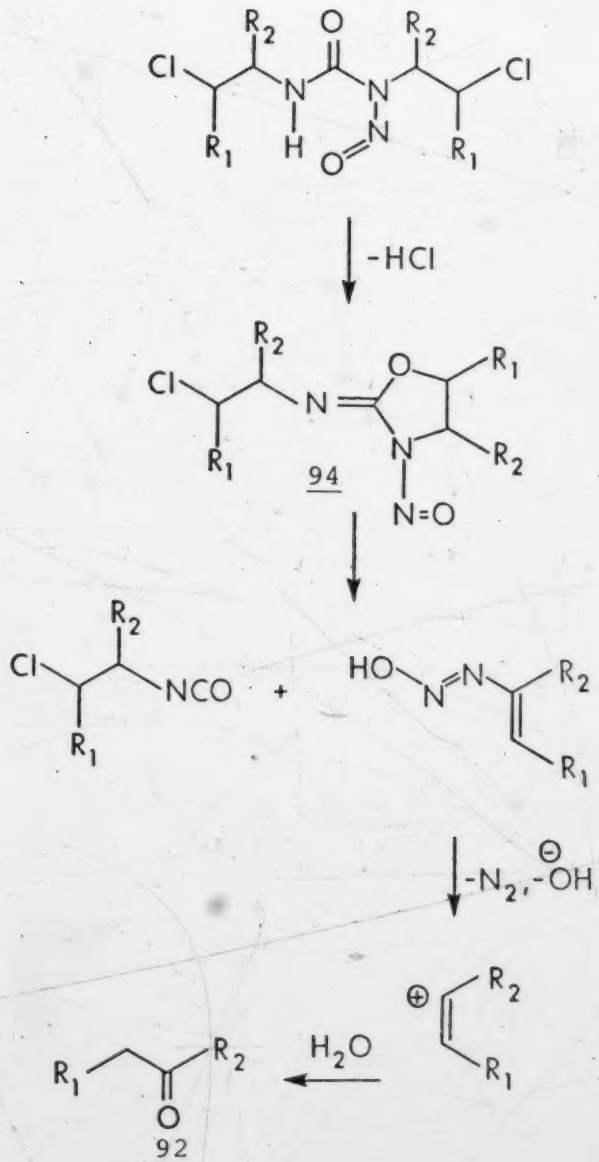


Figure 8. Decomposition *via* pathway A

(a) BCNU 5: $R_1 = R_2 = H$

(b) BCNU-β-Me: $R_1 = CH_3$, $R_2 = H$

(c) BCNU-α-Me: $R_1 = H$, $R_2 = CH_3$

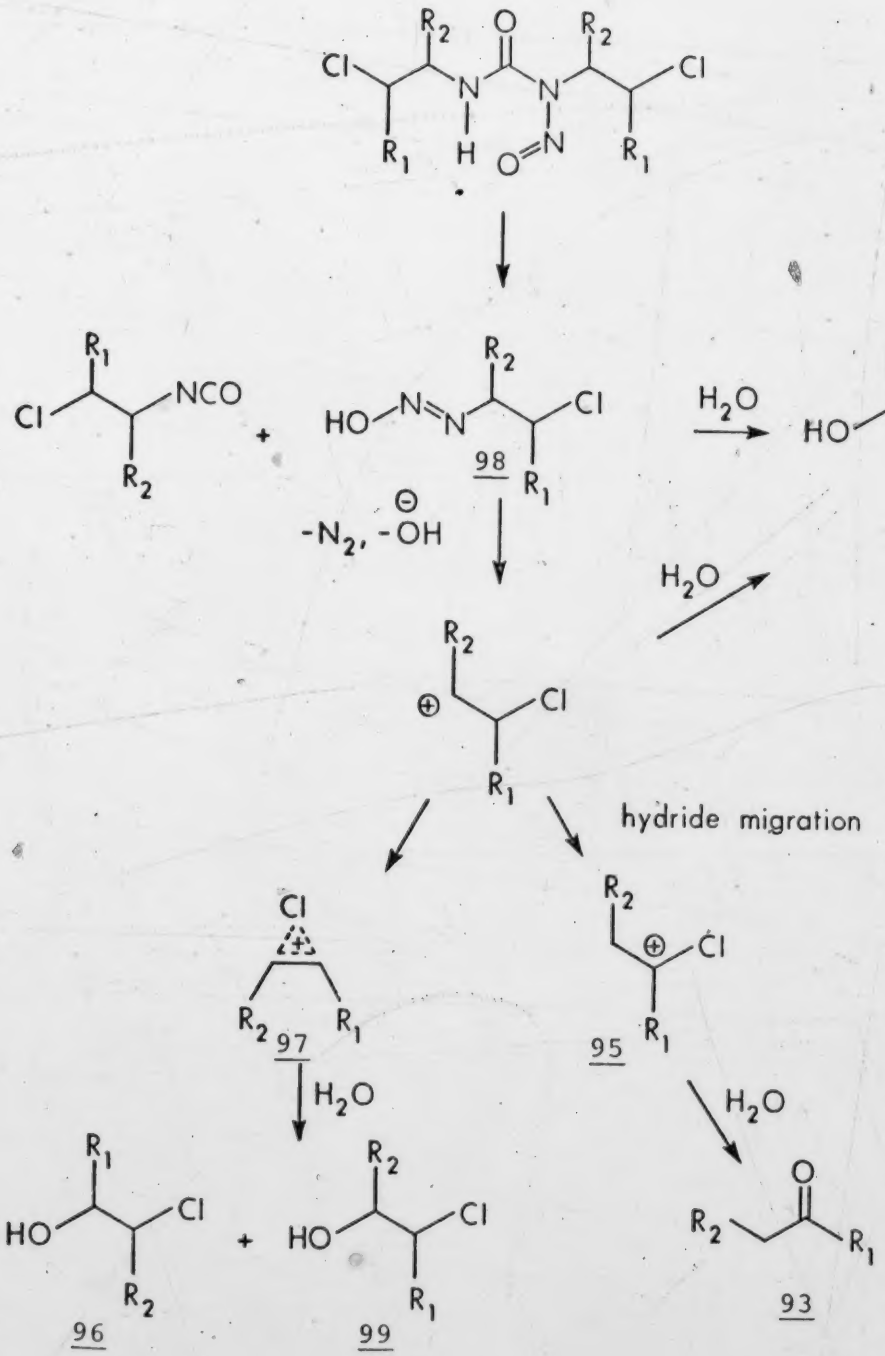


Figure 9. Decomposition *via* pathway B

(a) BCNU: $R_1 = R_2 = H$

(b) BCNU-β-Me: $R_1 = CH_3$, $R_2 = H$

(c) BCNU-α-Me: $R_1 = H$, $R_2 = CH_3$

methyl substituted analogues. Decomposition of BCNU-β-Me 60 *via* pathway A (Fig. 8) results in propionaldehyde 92b and decomposition *via* pathway B after hydride shift (Fig. 9) produces acetone 93b. BCNU-α-Me 61 results in acetone 92c by pathway A (Fig. 8) and propionaldehyde 93c by pathway B involving a hydride shift (Fig. 9).

The three nitrosoureas were allowed to decompose in aqueous solution buffered to pH 7.2 at 37°C for 24 h and the products separated by gas-liquid chromatography. The identified products are listed in Table 11. The absence of propionaldehyde from the decomposition of BCNU-β-Me 60 and the absence of acetone from the decomposition of BCNU-α-Me 61 indicates that decomposition *via* the cyclic 2-imino-N-nitrosooxazolidinone 94 (Fig. 8) is negligible for these two compounds in agreement with the deuterium labelling experiments of Brundrett[47] for BCNU 5.

The percentage of propionaldehyde produced in the decomposition of BCNU-α-Me 61 is nearly twice the percentage of acetone produced in the decomposition of BCNU-β-Me 60 (Table 6).

If pathway B (Fig. 9) involving hydride transfer to form the intermediate chlorocarbonium ion 95 followed by hydrolysis is the major pathway to the carbonyl containing decomposition products (acetaldehyde, acetone and propionaldehyde) as suggested by Brundrett,[47] for BCNU 5, then one would expect the relative amounts of propionaldehyde

Table 11

Decomposition of Substituted Haloethyl Nitrosoureas

Compound	Product
1,3-Bis(2-Chloroethyl)-1-nitrosourea (BCNU) 3	2-Chloroethanol (61%) Acetaldehyde (25%)
1,3-Bis(2-Chloropropyl)-1-nitrosourea (BCNU-β-Me) 60	1-Chloro-2-propanol (21%) 2-Chloro-1-propanol (21%) Acetone (21%) Propionaldehyde (0%)
1,3-Bis[1-(Chloromethyl)ethyl]-1-nitrosourea (BCNU-α-Me) 61	1-Chloro-2-propanol (30%) 2-Chloro-1-propanol (0%) Acetone (0%) Propionaldehyde (38%)

and acetone to be the reverse of that observed. BCNU-β-Me 60 (producing acetone) would form a secondary chlorocarbonium ion 95b (Fig. 9) after hydride transfer while BCNU-α-Me 61 (producing propionaldehyde) would form the less energetically favorable primary chlorocarbonium ion 95c (Fig. 9).

The decomposition of 1,3-bis(2-chloropropyl)-1-nitrosourea 60 (BCNU-β-Me) (Fig. 7) and 1,3-bis[1-(chloromethyl)ethyl]-1-nitrosourea 61 (BCNU-α-Me) (Fig. 7) also resulted in the chloropropanols listed in Table 11. The identification of both 1-chloro-2-propanol 96b (Fig. 9) and 2-chloro-1-propanol 99b (Fig. 9) in the decomposition of BCNU-β-Me 60 implicates the cyclic chloronium ion 97b (Fig. 9) as an intermediate. Since 1-chloro-2-propanol 96b would be the major product resulting from the hydrolysis of a methyl substituted cyclic chloronium ion 97b, the fact that both chloropropanols are produced in equivalent yields suggests a second source of 2-chloro-1-propanol 99b. S_N2 hydrolysis of the initially produced diazohydroxide 98b could account for additional 2-chloro-1-propanol 99b.

The absence of 2-chloro-1-propanol 96c in the decomposition of BCNU-α-Me 61 argues against the cyclic chloronium ion 97c (Fig. 9) intermediate in this case, since its hydrolysis should produce both chloropropanols 96c and 99c (Fig. 9).

The ratios of products produced in the decomposition of methyl substituted BCNU derivatives can be explained on the basis of recent theoretical calculations by Hehre and Hiberty[80] regarding the stabilities of the carbonium ions produced. The relative stabilities of the relevant carbonium ions are shown in Figure 10 which of course will be influenced by solvation effects.

Decomposition of BCNU-β-Me 60 by pathway B (Fig. 9) produces the 2-chloro-2-methylethyl carbonium ion 100 (Fig. 10) which can rearrange exothermically to produce the cyclic chloronium ion 101 (Fig. 10) or the 1-chloro-1-methylethyl carbonium ion 103. The former process results in the mixture of chloropropanols observed in Table 11 while the latter process could represent a major pathway to the acetone produced from BCNU-β-Me 60.

Decomposition of BCNU-α-Me 61 by pathway B (Fig. 9) results in the 2-chloro-1-methylethyl carbonium ion 104 (Fig. 10). In this case further rearrangement to the cyclic chloronium ion 101 (Fig. 10) is an unfavorable endothermic process. Thus, the only chloropropanol derivative observed after decomposition of BCNU-α-Me 61 is 1-chloro-2-propanol (Table 11). Hydride transfer to form the 1-chloro-2-methylethyl carbonium ion 105 (Fig. 10) is also an endothermic process and unlikely to be a major pathway to the propionaldehyde identified after the decomposition of BCNU-α-Me 61. These results implicate

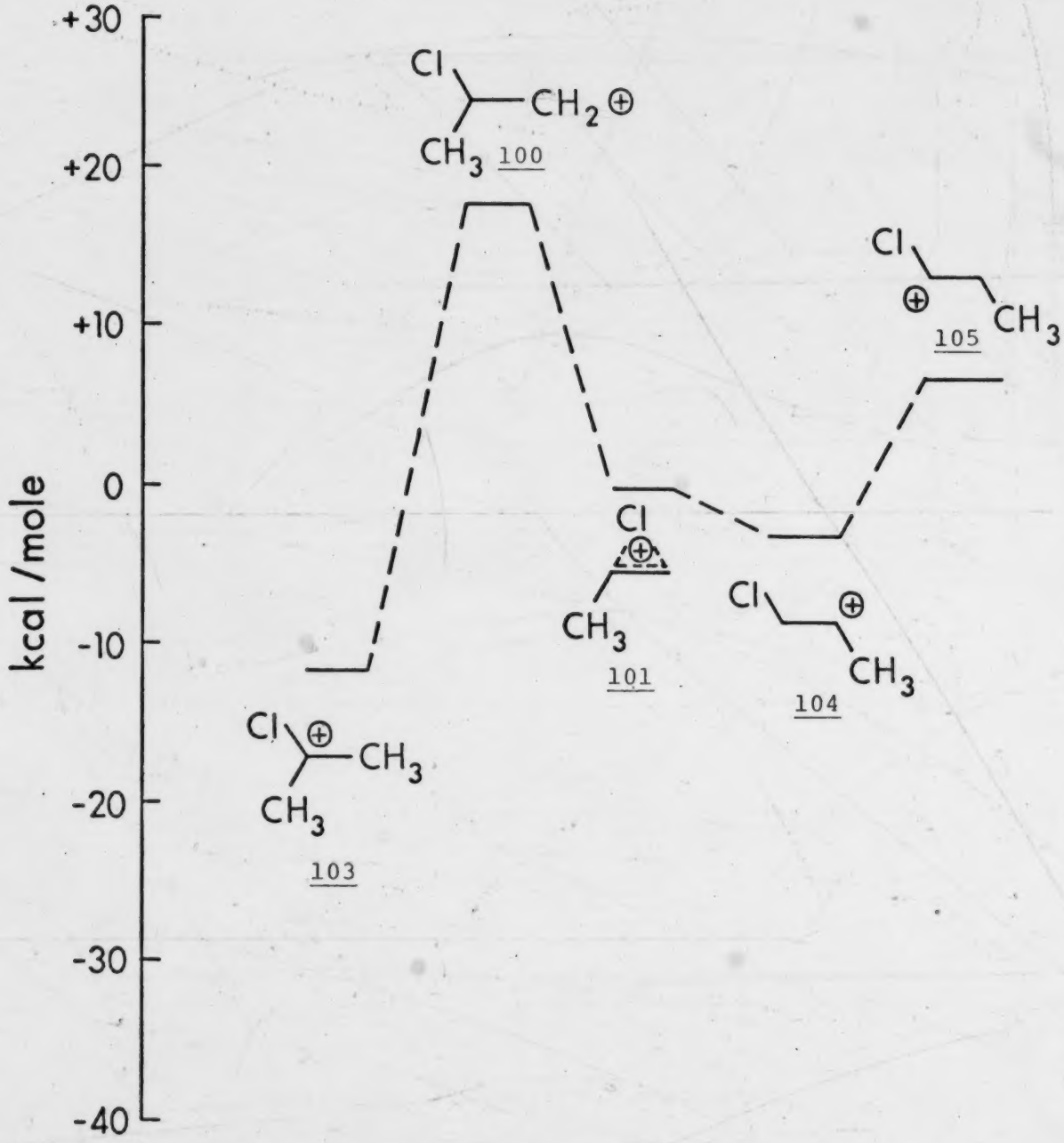
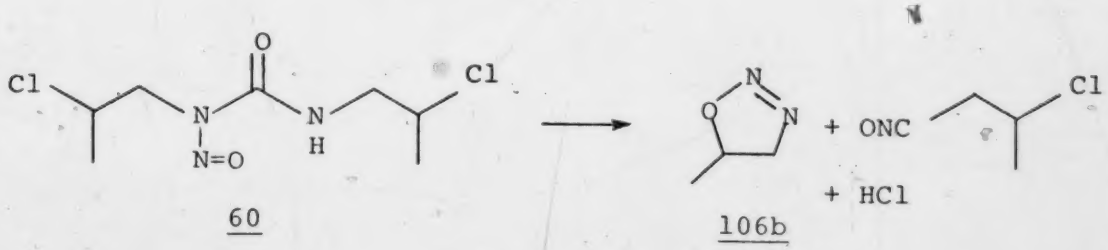


Figure 10. Energy map for monomethyl chloroethyl carbonium ions relative to the cyclic methyl substituted chloronium ion 101. Reproduced from W.J. Hehre and P.C. Hiberty, J. Am. Chem. Soc., 96, 2665 (1974).

the involvement of a third mechanistic pathway in addition to those involving the 2-imino-N-nitrosooxazolidinone and hydride transfer pathways previously discussed.

A 1,2,3-oxadiazoline 106 intermediate is in agreement with the experimental results presented. Initial ionization (proton loss) followed by isocyanate production and concomitant cyclization (with loss of halogen) could produce the oxadiazoline 106 (Fig. 11). The oxadiazoline 106 could undergo proton loss to produce acetaldehyde 107a (Fig. 11) (in accord with the deuterium labelling experiments of Brundrett[47]). Nucleophilic attack at the carbon bearing the nitrogen results in hydroxyethylated nucleophiles 108 (Fig. 11) (observed after the reaction of BFNU 26 and BCNU 5 with poly C[81]). As the leaving ability of the halogen increases in the series of 1,3-bis(2-haloethyl)-1-nitrosoureas, 1,2,3-oxadiazoline 106 formation would be favored and result in greater percentages of acetaldehyde as was observed (Table 10).

Decomposition of BCNU-β-Me 60 *via* the methyl substituted 1,2,3-oxadiazoline 106b would involve intramolecular substitution of the chlorine at a secondary carbon center:



60 106b

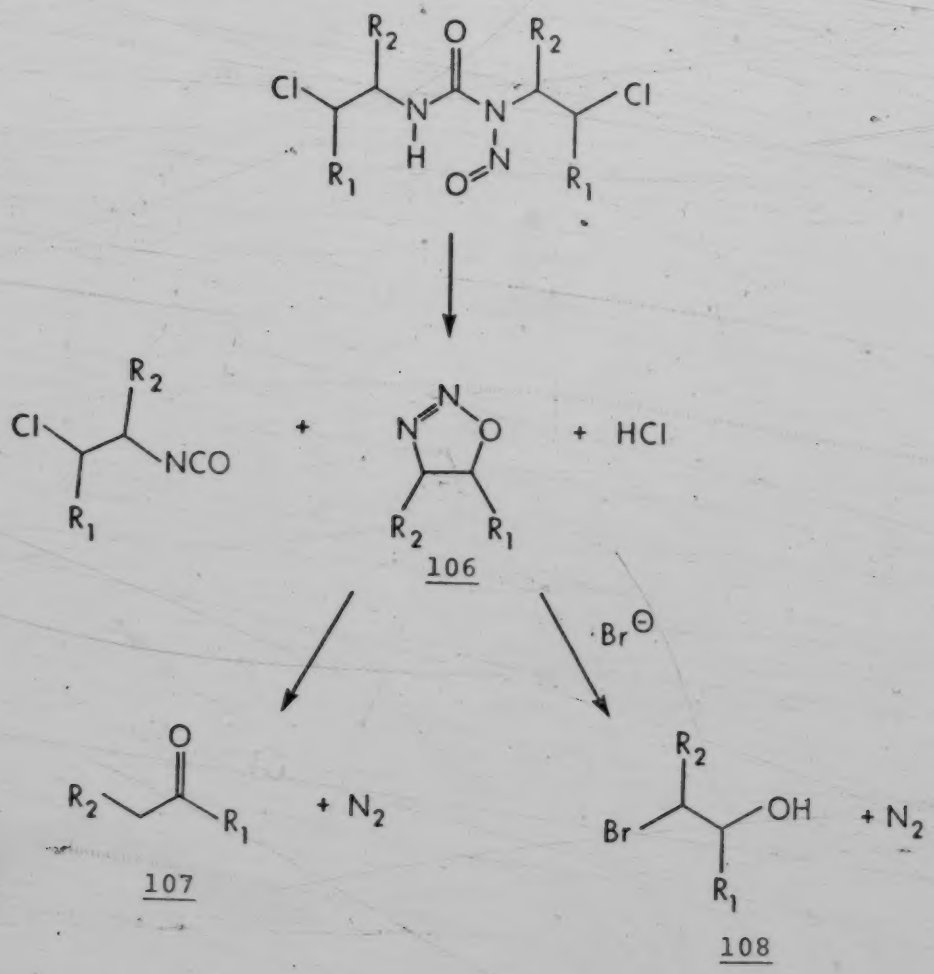


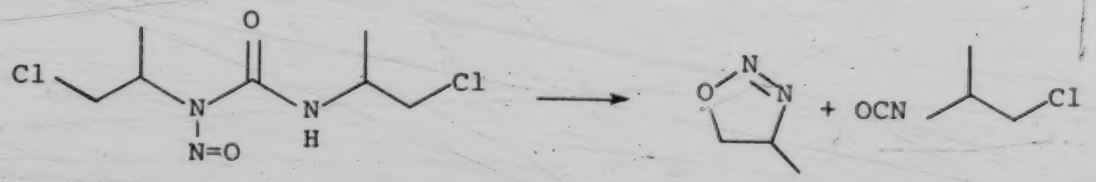
Figure 11. Decomposition *via* pathway C

(a) BCNU: $R_1 = R_2 = H$

(b) BCNU-β-Me: $R_1 = CH_3$, $R_2 = H$

(c) BCNU-α-Me: $R_1 = H$, $R_2 = CH_3$

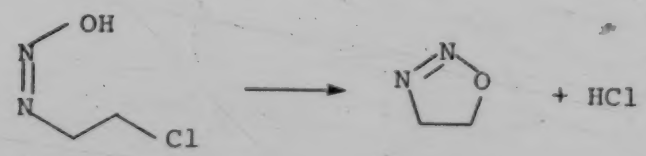
In the case of BCNU-α-Me 61, the cyclization occurs at a primary carbon center:



61 106c + HCl

Since the latter reaction is energetically more favorable one would expect a higher percentage of the carbonyl containing compound as was observed (Table 11).

Montgomery has recently suggested[82] that the 2-chloroethyl diazohydroxide 76 initially produced upon decomposition of 2-haloethylnitrosoureas could cyclize and result in a 1,2,3-oxadiazoline 106a. Such a cyclization

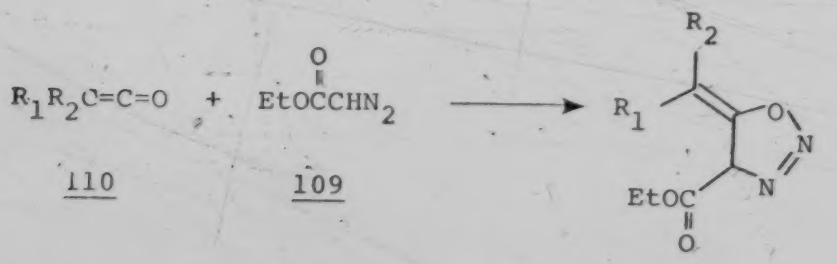


76 106a

would, of course, require the *syn*-2-chloroethyl diazohydroxide 76. *Syn*-diazotates 17 (Fig. 2) have been observed[63] to undergo rapid aqueous decomposition (by

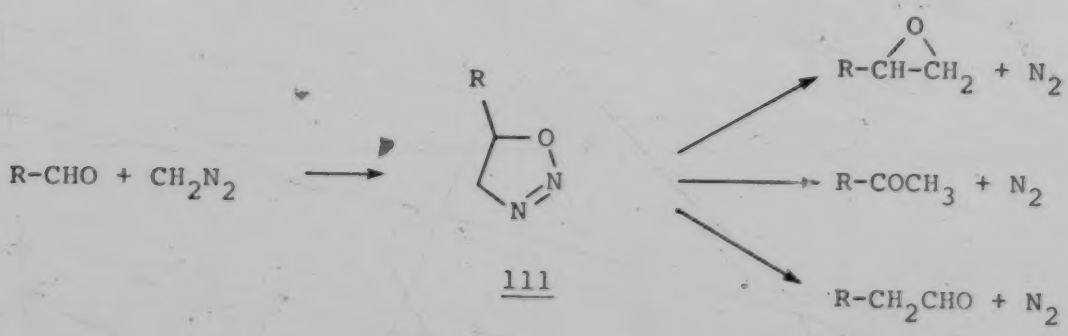
protonation to form the diazohydroxide followed by elimination of hydroxide - see Figure 2) with the production of diazoalkane 18 and/or carbonium ion 19 species. Such an energetically favorable pathway would be expected to compete favorably with intramolecular cyclization to produce the 1,2,3-oxadiazoline 106a.

1,2,3-Oxadiazolines have not been well characterized to date. They have been suggested[83] to result from the reaction of diazoacetic esters 109 with ketenes 110 but not conclusively proven. 1,2,3-oxadiazoline inter-



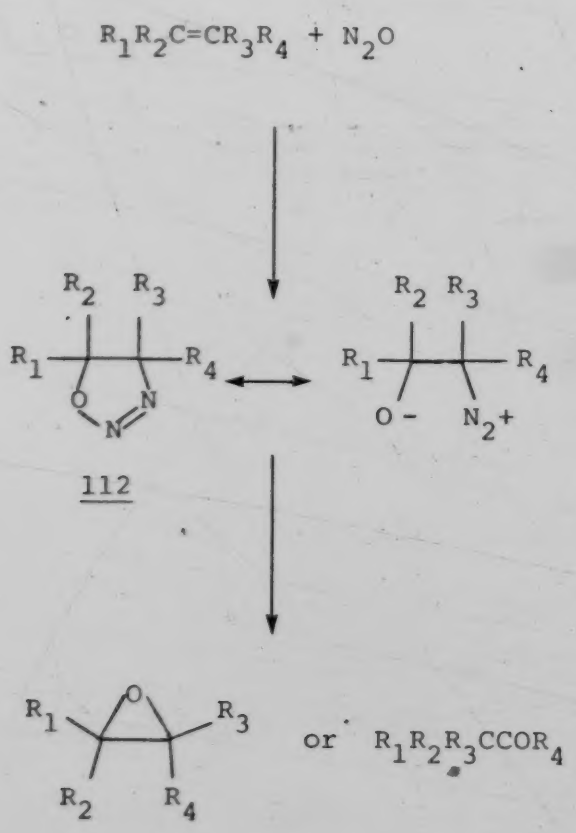
110 109

mediates 111 were suggested as intermediates in the reaction of diazomethane with aldehydes.[84-86] It has also been



111

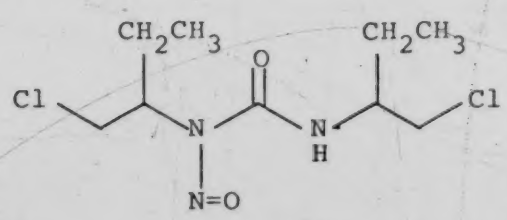
suggested[87] that the reaction of nitrous oxide with olefins involves a 1,2,3-oxadiazoline 112.



Further evidence for the intermediacy of a 1,2,3-oxadiazoline in the decomposition of 2-haloethylnitrosoureas was obtained by an additional experiment. It appeared that should a 1,2,3-oxadiazoline 106a be formed during the decomposition of BCNU 5, it might be susceptible to nucleophilic attack at the carbon bearing the nitrogen and produce hydroxyethylated products 108a as shown for the bromide nucleophile in Figure 11.

In an attempt to trap an oxadiazoline intermediate 106 with a nucleophile, the decomposition of BCNU 5 was carried out in an aqueous saturated sodium bromide. GC-mass spectral analysis of the reaction solution indicated the existence of significant amounts of two new products: 1-bromo-2-chloroethane and 2-bromoethanol. A control experiment, which involved the incubation of 2-chloroethanol in an aqueous saturated sodium bromide solution, indicated that 2-bromoethanol did not result from bromide substitution for chloride in the 2-chloroethanol produced. The formation of 1-bromo-2-chloroethane has been observed[67] by a similar experiment involving BCNU 5 and can be explained to result from bromide ion attack of a 2-chloroethyl diazohydroxide, 2-chloroethyl carbonium ion or cyclic chloronium ion (Fig. 9). The most reasonable explanation for the formation of 2-bromoethanol involves bromide ion attack on a 1,2,3-oxadiazoline intermediate 106.

BCNU-α-Me 61 (Fig. 7) hydrolyzes at a rate three times faster than BCNU 5 (Table 6). A similar relationship has been reported by Wheeler[15] for 1,3-bis[1-(chloromethyl)propyl]-1-nitrosourea 113 ($t_{1/2}$ 14.7 min) and BCNU 5 ($t_{1/2}$ 43 min). Conversely, BCNU-β-Me 60 (Fig. 7)



113

decomposes at a rate comparable to BCNU 5 (Table 6) which was also reported by Wheeler[15] (BCNU-β-Me 61 $t_{1/2}$ 41 min). It is evident that alkyl substitution on the α-carbon (adjacent to the urea nitrogens) increases the rate of aqueous decomposition relative to the unsubstituted derivative (BCNU 5). Whether such substitution results in steric interactions with the carbonyl and nitroso functions which permit an energetically favorable pathway to a 1,2,3-oxadiazoline 106 intermediate and observed carbonyl containing decomposition products cannot be determined at this time.

Conclusions

The proposed pathways for the decomposition of 2-haloethylnitrosoureas 114 are shown in Figure 12. Proton loss by ionization or transfer to the nitroso group (pathway B) initiates decomposition. Pathway C and/or C' (*via* the oxadiazoline 106) will be favored when X is a good leaving group and is bonded to a primary center. The

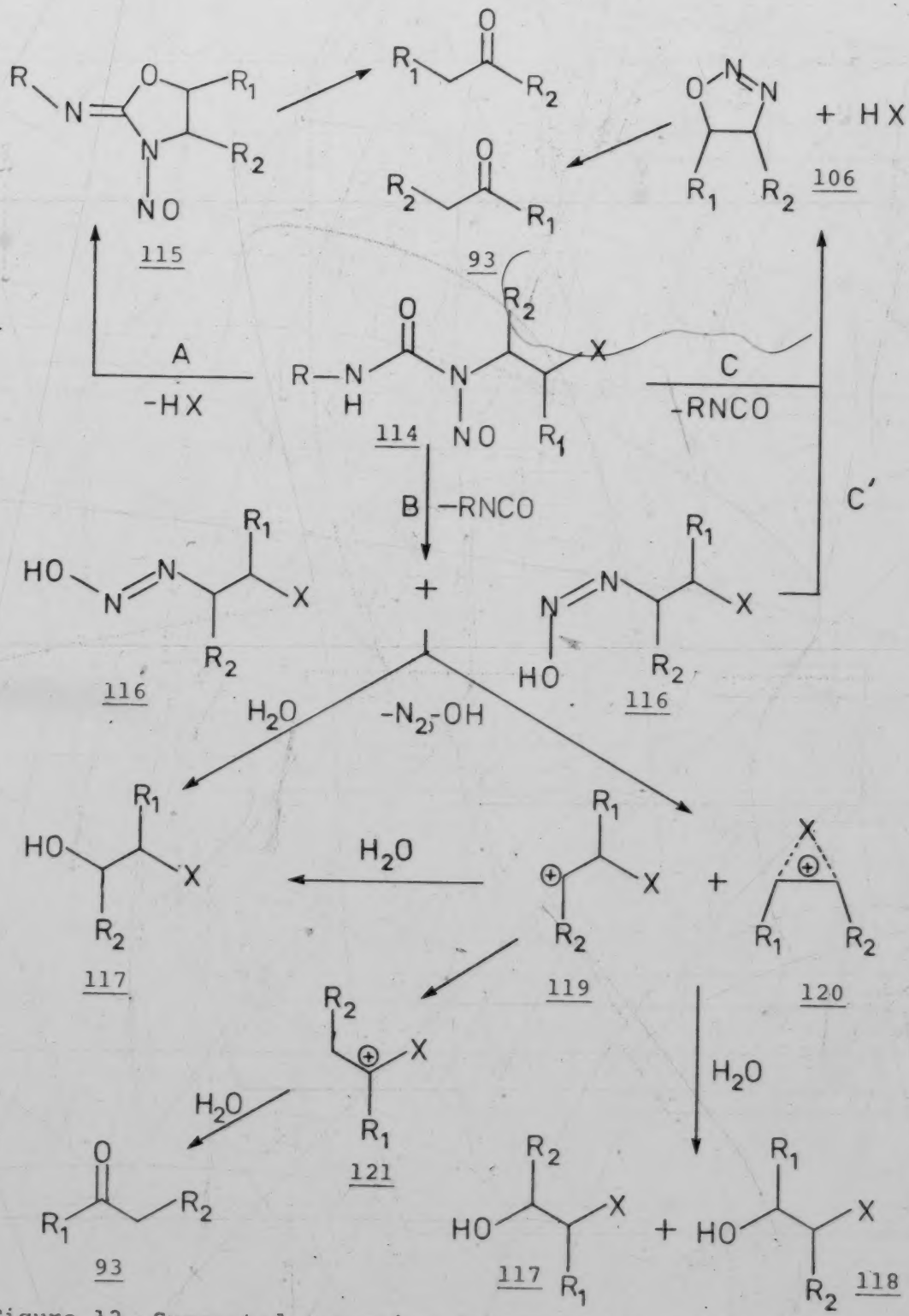


Figure 12. Suggested competing decomposition pathways for 2-haloethylnitrosoureas.

oxadiazoline 106 accounts for carbonyl containing compounds 93 as well as hydroxyethylated nucleophiles. Vinyl alkylating agents may be produced from an oxadiazoline intermediate, however, it has previously been shown[47] that vinyl alkylating agents only represent a minor decomposition pathway. Pathway A (*via* the 2-imino-N-nitroso-oxazolidinone 115) does not appear to be significant when X is chlorine but may be a contributing pathway with superior leaving groups.

Pathway B (*via* the 2-haloethyl diazohydroxide 116) results in the haloethyl alcohols 117, 118 produced either by S_N2 hydrolysis of the diazohydroxide 116 or reaction of the subsequent carbonium ion 119 and/or cyclic haloethonium ion 120 with water. When X is a polarizable group, and when it is bonded to a secondary carbon center, hydride migration to form the halocarbonium ion 121 could be an energetically favorable pathway to carbonyl containing decomposition products 93.

Experimental

Throughout this work melting points were determined on a Fisher-Johns apparatus and are uncorrected. The ir spectra were recorded on a Nicolet 7199 F.T. spectrophotometer, and only the principal, sharply defined peaks are reported. The nmr spectra were recorded on Perkin Elmer 90 and Varian HA-100 analytical spectrometers. The spectra were measured on approximately 10-15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in ppm from the reference. Mass spectra were determined on an Associated Electrical Industries MS-9 double focussing high resolution mass spectrometer. The ionization energy, in general, was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin layer chromatography. Microanalyses were carried out by Mrs. D. Mahlow of this department. In the work-up procedures reported for the various syntheses described, solvents were removed with a rotary evaporator under reduced pressure unless othewise stated.

1,3-Bis(2-fluoroethyl)-1-nitrosourea 26 and 1-(β-D-glucopyranosyl)-3-(2-chloroethyl)-3-nitrosourea (GANU) 88 were gifts from Dr. Harry B. Wood Jr., Division of Cancer Treatment, National Cancer Institute, Washington,

D.C. Chlorozotocin 86 was obtained from Dr. Gerald Goldenberg, Manitoba Institute of Cell Biology, Winnipeg, Manitoba. N-methyl-N'-nitro-N-nitrosoguanidine 1 was purchased from Aldrich and streptozotocin 87 from Calbiochem. Compounds not previously known are described below in detail, compounds prepared by literature procedures are so noted.

N-Methyl-N-nitrosourea 2.

This compound was prepared according to the method of Vogel.[88] 2.4 g (53% yield) mp 123-124°C (lit.[88] 123-124°C).

N-Ethyl-N-nitrosourea 10.

This compound was prepared according to the method of Mirvish.[89] 2.8 g (62% yield), mp 98-100°C (lit.[89] 99-100°C). Pmr ($CDCl_3$) δ 1.0 (t, 3H, CH_3), 3.8 (q, 2H, CH_2), 7.0 (s, 2H, exchangeable).

2-Haloethyl nitrosoureas.

The following compounds were prepared according to the methods described by Montgomery *et al.*[2,3] Compound, Yield, mp (lit. ref., mp), Pmr (solvent).

1,3-Bis(2-chloroethyl)-1-nitrosourea 5 54%, 30-32°C (3, 30-32°C), Pmr ($CDCl_3$) δ 3.5 (t, 2H, CH_2), 3.8 (m, 4H, CH_2), 4.2 (t, 2H, CH_2), 7.4 (d, 1H, exchangeable).

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea 6, 76%, 88-90°C (3, 90°C), Pmr ($CDCl_3$) δ 1.2-2.2 (m, 10H, CH_2),

3.5 (t, 2H, CH_2), 3.9 (m, 1H, CH), 4.2 (t, 2H, CH_2), 6.8 (d, 1H, exchangeable).

1-(2-Chloroethyl)-1-nitrosourea 3, 25%, 75-77°C, (2, 75-78°C), Pmr ($CDCl_3$) δ 3.5 (t, 2H, CH_2), 4.2 (t, 2H, CH_2), 5.7-7.0 (m, 2H, exchangeable).

1,3-Bis(2-bromoethyl)-1-nitrosourea 62, 67%, 34-36°C, (3, 36-38°C), Pmr ($CDCl_3$) δ 3.3 (t, 2H, CH_2), 3.5 (t, 2H, CH_2), 3.9 (q, 2H, CH_2), 4.2 (t, 2H, CH_2), 7.3 (d, 1H, exchangeable).

1,3-Bis(2-iodoethyl)-1-nitrosourea 63, 60%, 57-59°C, (3, 58-60°C), Pmr ($CDCl_3$) δ 3.1 (t, 2H, CH_2), 3.4 (t, 2H, CH_2), 3.9 (q, 2H, CH_2), 4.2 (T, 2H, CH_2), 7.3 (d, 1H, exchangeable).

1-(2-Fluoroethyl)-3-cyclohexyl-1-nitrosourea 64, 65%, 37-38°C (3, 34-37°C), Pmr ($CDCl_3$) δ 1.1-2.1 (m, 10H, CH_2), 3.9 (m, 1H, CH), 4.0-4.4 (m, 2H, CH_2F), 4.7 (t, 2H, CH_2), 6.8 (d, 1H, exchangeable).

1-(2-Bromoethyl)-3-cyclohexyl-1-nitrosourea 65, 83%, 75-75.5°C (3, 75°C), Pmr ($CDCl_3$) δ 1.2-2.2 (m, 10H, CH_2), 3.3 (t, 2H, CH_2), 3.9 (m, 1H, CH), 4.2 (t, 2H, CH_2), 6.8 (d, 1H, exchangeable).

1-(3-Chloropropyl)-1-nitrosourea 57.

Sodium cyanate (675 mg, 10.0 mmole) was added to 1.0 g (7.7 mmole) of 3-chloropropylamine hydrochloride in 10 ml of water and the mixture stirred mechanically

overnight. After chilling the resulting precipitate was collected and recrystallized from chloroform:petroleum ether affording 1-(3-chloropropyl)urea 800 mg (76% yield) m.p. 98-99°C (lit.[90] 98-99°C).

1-(2-Chloropropyl)urea (800 mg, 5.9 mmole) was nitrosated in 1 ml of 98% formic acid at 0-5° using 500 mg (7.2 mmole) of sodium nitrite. After stirring the mixture for 1 hr, 1 ml of cold water was added cautiously and stirring continued for 30 min. The mixture was extracted with chloroform, washed with H_2O, dried ($MgSO_4$) and the solvent removed. The residual solid was recrystallized from ether/petroleum ether to give 1-(3-chloropropyl)-1-nitrosourea 560 mg (58% yield) m.p. 78-79°C.

Anal. Calcd. for $C_4H_8ClN_3O_2$: (m.w. 165.0305); C, 29.01; H, 4.88; N, 25.38; Cl, 21.41. Found (165.0313, mass spectrum) C, 29.13; H, 4.83; N , 25.15; Cl, 21.51. Pmr ($CDCl_3$) δ 1.9 (m, 2H, CH_2); 3.4 (t, 2H, CH_2); 4.0 (t, 2H, CH_2); 5.1-7.0 (m, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3380 (N-H); 1735 (C=O); 1480 cm^{-1} (N=O).

1-(4-Chlorobutyl)-1-Nitrosourea 58.

Potassium cyanate (310 mg, 4.0 mmol) was added to 600 mg (4.0 mmol) of 4-chlorobutylamine hydrochloride in 5 ml of water and the mixture stirred overnight. After chilling the resulting precipitate was collected, and air dried, 520 mg (85% yield). This crude product, although

slightly contaminated with the starting amine hydrochloride was found suitable for nitrosation. 200 mg of crude 4-chlorobutylurea in 1 ml of 98% formic acid at 0°C was treated with 150 mg of sodium nitrite added in portions over 20 min. After an additional 30 min of stirring at 0°C, 5 ml of water was cautiously added. The pale yellow solid was collected, dried and recrystallized from ether/petroleum ether 140 mg (59% yield) m.p. 64-65°C.

Anal. Calcd. for $C_5H_{10}ClN_3O_2$ (m.w. 179.0461): C, 33.43; H, 5.62; N, 23.40; Cl, 19.74. Found (179.0460, mass spectrum): C, 33.50; H, 5.64; N, 23.70; Cl, 19.96. Pmr ($CDCl_3$) δ 2.6 (m, 4H, CH_2); 3.5 (t, 2H, CH_2); 3.8 (t, 2H, CH_2); 5.8 (s, 1H, exchangeable); 6.8 (s, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3300, 3220 (N-H); 1730 (C=O); 1480 cm^{-1} (N=O).

1-(5-Chloropentyl)-1-nitrosourea 59.

This compound was prepared by the same method as 1-(4-chlorobutyl)-1-nitrosourea. The nitrosation of a crude 250 mg sample of 1-(5-chloropentyl)urea gave the nitrosourea as a pale yellow solid. 225 mg (64% yield) m.p. 65-66°C.

Anal. Calcd. for $C_6H_{12}ClN_3O_2$ (m.w. 193.0614): C, 37.21; H, 6.26; N, 21.70; Cl, 18.31. Found (193.0616, mass spectrum): C, 37.15; H, 6.23; N, 21.86; Cl, 18.38. Pmr ($CDCl_3$) δ 1.3-1.9 (m, 6H, CH_2); 3.5 (t, 2H, CH_2); 3.8 (t, 2H, CH_2); 5.9 (s, 1H, exchangeable); 6.8 (s, 1H,

exchangeable). Ir ν_{max} ($CHCl_3$) 3400, 3240 (N-H); 1770 (C=O); 1480 cm^{-1} (N=O).

1,3-Bis(2-chloropropyl)-1-nitrosourea 60.

This compound was prepared according to the method of Montgomery *et al.*[3] 120 mg (56% yield), oil (lit.[3] oil), Pmr ($CDCl_3$) δ 1.4 (t, 3H, CH_3); 1.5 (t, 3H, CH_3); 3.4-4.4 (m, 6H, $CH+CH_2$), 7.4 (t, 1H, exchangeable).

1,3-Bis[1-(chloromethyl)ethyl]urea 40.

1-(Chloromethyl)ethyl isocyanate,[91] was added to a solution of 1 ml triethylamine in 9 ml of H_2O at 0°C and the mixture stirred for 2 h. The white solid was collected and purified by recrystallization from $CHCl_3$/Pet. ether, 275 mg (estimated 40% yield) m.p. 117-119.

Anal. Calcd for $C_7H_{14}Cl_2N_2O$ (m.w. 212.0483): C, 39.45; H, 6.63; N, 13.15; Cl, 33.27. Found (212.0490, mass spectrum): C, 39.47; H, 6.54; N, 13.15; Cl, 33.23. Pmr ($CDCl_3$) δ 1.2 (d, 6H, CH_3); 3.6 (m, 4H, CH_2); 4.2 (m, 2H, CH); 4.6 (d, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3000 (N-H); 1705 (C=O) cm^{-1}.

1,3-Bis[1-(chloromethyl)ethyl]nitrosourea 61.

To 100 mg of 1,3-bis[1-chloromethyl)ethyl]urea in 2 ml of 98% HCOOH at 0°C was added during 2 hr 200 mg of $NaNO_2$. The mixture stirred an additional 2 hr at 0°C. 5 ml of H_2O was then cautiously added and the resulting

solution extracted with ether. The ether extract was washed with H_2O dried ($MgSO_4$) and the ether removed to yield a pale yellow oil which could be crystallized from pet. ether, 60 mg (55% yield) m.p. 30-31°C.

Anal. Calcd. for $C_7H_{13}Cl_2N_3O_2$ (m.w. 241.0385): C, 34.87; H, 5.42; N, 17.43; Cl, 29.40. Found (241.0389, mass spectrum): C, 34.72; H, 5.51; N, 17.22; Cl, 29.58. Pmr ($CDCl_3$) δ 1.3 (d, 3H, CH_3); 1.4 (d, 3H, CH_3); 3.5-4.0 (m, 4H, CH_2); 4.4 (m, 1H, CH); 5.1 (m, 1H, CH), 7.0 (s, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3300 (N-H); 1695 (C=O); 1505 (N=O) cm^{-1}.

3-Cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea 66.

Cyclohexyl isocyanate (2.5 g, 20.0 mmole) was added to 1.2 g (20.0 mmole) of ethanolamine in toluene at ambient temperature. After 4 hours 2.9 g of the crude 3-cyclohexyl-1-(2-hydroxyethyl)urea was collected. A 500 mg (2.2 mmole) protion of the urea was dissolved in 5 ml of 98% formic acid at 0-5°C and 300 mg (4.0 mmole) of sodium nitrite added slowly over a 30 min period maintaining a temperature of 0-5°C. After stirring for 30 min 10 ml of cold water was added cautiously. The mixture was extracted with chloroform, the extract washed with water, dried ($MgSO_4$) and the solvent removed. The residue was recrystallized from chloroform/petroleum ether affording 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea 310 mg (54% yield) m.p. 49-51°C.

Anal. Calcd. for $C_9H_{17}N_3O_3$ (m.w. 215.1270): C, 50.24; H, 7.98; N, 19.54. Found (215.1265, mass spectrum), C, 50.21; H, 8.00; N, 19.58. Pmr ($CDCl_3$) δ 1.2-2.2 (m, 10H, CH_2); 2.7 (s, 1H, exchangeable); 3.6 (t, 2H, CH_2); 3.85 (m, 1H, CH); 4.16 (t, 2H, CH_2); 6.9 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3490 (OH; 3370 (NH); 1705 (C=O); 1480 cm^{-1} (N=O).

3-Cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea 67.

Cyclohexyl isocyanate (3.0 g, 24.0 mmole) was added dropwise to 2.0 g (24.0 mmole) of 2-methoxyethylamine in benzene at room temperature. After stirring for 4 hr, 3.8 g of the crude 3-cyclohexyl-1-(2-methoxyethyl)urea was collected. A 500 mg portion of the urea was nitrosated by the same procedure described above giving 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea as a pale yellow oil which crystallized from petroleum ether upon chilling 300 mg (52% yield) m.p. 23°.

Anal. Calcd. for $C_{10}H_{19}N_3O_3$ (m.w. 229.1426): C, 52.42; H, 8.37; N, 18.34. Found (229.1426, mass spectrum), C, 52.74; H, 8.40; N, 18.34. Pmr ($CDCl_3$) δ 1.2-2.2 (m, 10H, CH_2); 3.3 (s, 3H, CH_3); 3.4 (t, 2H, CH_2); 3.7-4.1 (m, 1H, CH); 4.1 (t, 2H, CH_2); 6.9 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3350 (NH); 1735 (C=O); 1490 cm^{-1} (N=O).

1-(2-Chloroethyl)-3,3-dimethyl-1-nitrosourea 70.

This compound was prepared according to the method of Colvin *et al.*[11] 110 mg (54% yield), oil (lit.[11] oil). Pmr ($CDCl_3$) δ 3.2 (s, 6H, CH_3); 3.6 (t, 2H, CH_2); 4.2 (t, 2H, CH_2).

N-Nitroso-2-oxazolidinone 89.

This compound was prepared according to the method of Newman and Kutner.[79] 380 mg (68% yield) m.p. 48-50°C (lit.[79] 50-53°C). Pmr ($CDCl_3$) δ 3.5 (t, 2H, CH_2); 4.1 (t, 2H, CH_2).

Methods

Polarographic Determination of Decomposition Rates for Nitrosoureas.

The Princeton Applied Research (PAR) Model 174A polarograph and 9300-9301 polarographic cell were used in a three electrode configuration which included an aqueous saturated calomel reference electrode (SCE), to which all potentials in this paper are relative, a platinum counter electrode, and a dropping mercury electrode (DME) with a controlled 2 s drop time. The temperature in the cell was maintained at 37.5 ± 0.2°C by circulation of thermostatted water unless otherwise indicated. The resulting curves were recorded on a Houston 2000 X-Y recorder. The sample solutions were

buffered at pH 7.1 with 0.01 M potassium phosphate buffer in 0.01 M KCl supporting electrolyte. The pH value of the sample solutions were measured with an Accumet Model 520 pH meter before each run.

For compounds which showed extremely low solubility in aqueous solution, 5% acetonitrile aqueous solution was used; in some cases differential pulse polarography of the aqueous solution was sufficiently sensitive and this was used whenever possible. All solutions were deareated with purified nitrogen for 10 min before a run and blanketed with it during the run. The Arrhenius parameters for the 1,3-bis(2-haloethyl)-nitrosoureas were determined from the rate data at different temperatures.

Product Decomposition Studies

(a) Decomposition of 2-Haloethylnitrosoureas.

The decompositions were carried out at pH 7.2, 37°. One milliliter of a 40 mM nitrosourea solution was allowed to decompose in a sealed tube for 24 hr. The solutions were then cooled to 4°, the sealed tube was opened and immediate gas chromatographic (GC) analysis of the solution was undertaken. GC analyses were performed on a Hewlett-Packard Model 5830 A temperature programmable research chromatograph equipped with a flame ionization detector. Samples were injected onto a 2 m 6.5 mm-o.d.

column of 10% Carbowax on Chromosorb W. The column was heated at 90°C for 4 min after injection; a heating rate of 20°/min was then maintafned until the column temperature reached 120°C; this temperature was maintained until all volatile products had been swept from the column. Identification of acetaldehyde and haloethanol was done using retention times of authentic reference samples.

Retention times of authentic samples: acetaldehyde, 0.7 min; 2-fluoroethanol, 1.7 min; 2-chloroethanol, 4.6 min; 2-bromoethanol, 8.3 min; 2-iodoethanol, 12.4 min.

(b) Decomposition of BCNU and methyl substituted derivatives

The decompositions were carried out at pH 7.2, 37°C. One milliliter of a 40 mM nitrosourea solution was allowed to decompose in a sealed tube for 24 hr. The solutions were then cooled to 4°C, the sealed tube was opened, and immediate gas chromatographic (GC), analysis of the solution was undertaken.

GC analyses were performed on a Hewlett-Packard Model 5830 A temperature programmable research chromatograph equipped with a flame ionization detector. Samples were injected onto a 6-m, 6.5-mm-o.d. column of 10% Carbowax on Chromosorb W. The column was heated at 50°C for acetaldehyde, acetone and propionaldehyde measurements and at 150°C for chloroethanol, 2-chloro-1-propanol and 1-chloro-2-propanol measurements. Identification was done using retention times of authentic samples.

Retention times of authentic samples: propionaldehyde 4.1 min; acetone, 4.5 min; 1-chloro-2-propanol, 6.2 min; 2-chloro-1-propanol, 7.4 min.

(c) Decomposition of BCNU 5 in saturated NaBr.

The decomposition was carried out at pH 7.2, 37°C in a saturated sodium bromide solution. Once milliliter of a 40 mM BCNU 5 solution was allowed to decompose in a sealed tube for 24 hr. G.C. analysis was done as in (b). Identification was done using retention times of authentic samples and by G.C.-mass spectral analysis. Two new products were identified:

(1) 1-bromo-2-chloroethane, retention time 4.5 min. Mass spectral data: m/e (relative intensity) [142 (5.3), 144(6.9); M^+, $BrCH_2CH_2Cl$], [107(3.1), 109(2.3); M^+-Cl, $BrCH_2CH_2^+$], [63(100), 65(33); M^+-Br, $+CH_2CH_2Cl$].

(2) 2-bromoethanol, retention time 13.5 min. Mass spectral data: m/e (relative intensity) [124(4.7), 126(4.8); M^+, $BrCH_2CH_2OH$], [45(74), M^+-Br, $+CH_2CH_2OH$], [31(100); M^+-CH_2Br, $CH_2=\overset{+}{O}H$].

A control experiment was run using 2-chloroethanol in place of the nitrosourea. Incubation of the mixture followed by GC analysis indicated that less than 2% of the 2-chloroethanol could be converted to 2-bromoethanol under these conditions.

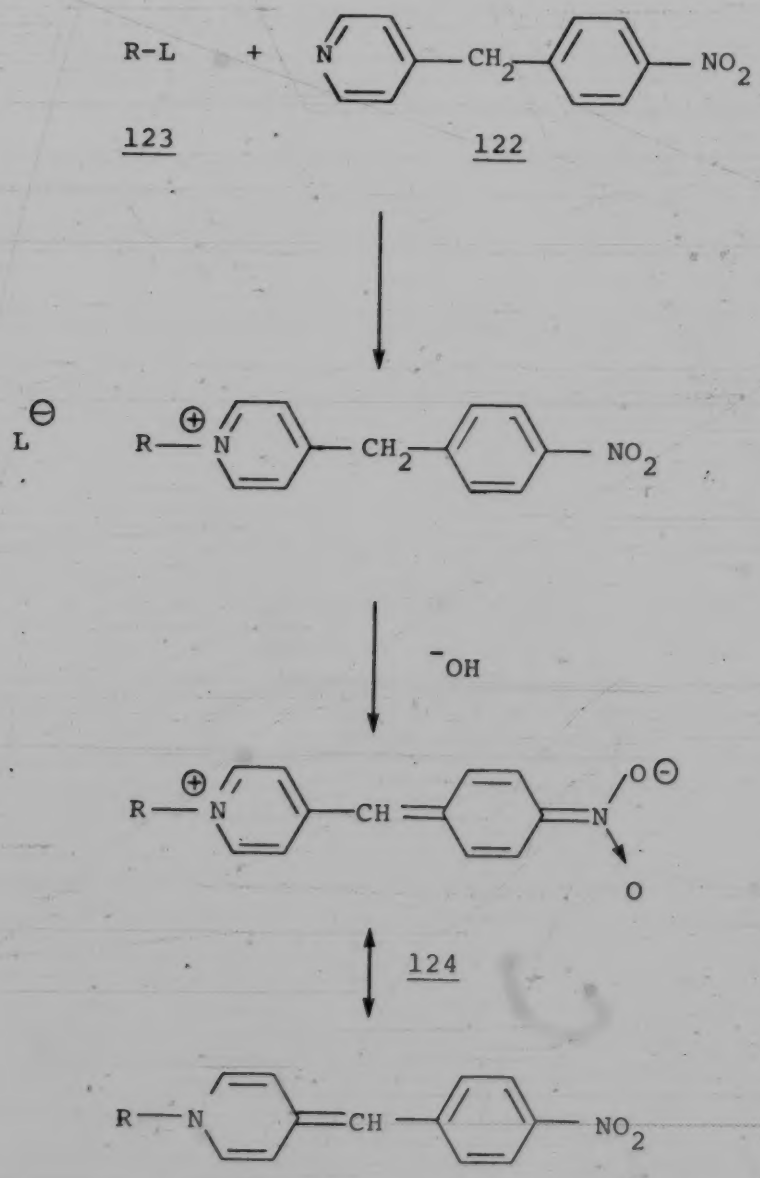
CHAPTER THREE

ALKYLATION AND INTERSTRAND CROSS-LINKING OF DNA BY NITROSOUREAS

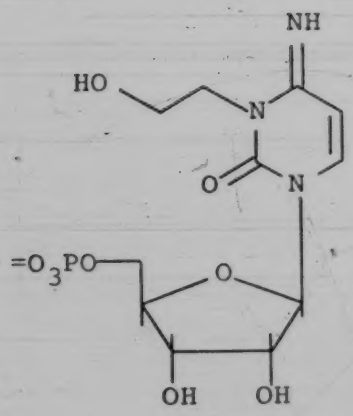
ENU 10 and MNU 2 are known mutagenic and carcinogenic compounds which alkylate nucleic acids as reported by a number of investigators.[12,21,92] Many of the potentially nucleophilic sites in the DNA molecule (see Chapt. I) have been observed[92] to undergo alkylation by nitrosoureas. While MNU 2 reacts as a typical S_N2 alkylating agent, producing a relatively large amount of 7-methylguanosine,[93,94] ENU 10 appears to produce increased amounts of the minor alkylation products including O^6-ethylguanosine[25,95-97] and ethyl phosphotriesters.[95-97] Similar differences between ethylating and methylating agents have been observed for sulfates[95,98] and alkyl sulfonates,[95,98] which may reflect preferences toward S_N1 and S_N2 reactivity for ethyl and methyl alkylating agents respectively. That the 2-fluoroethyl- and 2-chloroethylnitrosourea derivatives exhibit alkylating activity was first reported by Wheeler and Chumley[99] using the 4-(p-nitrobenzyl)pyridine assay.

The use of 4-(p-nitrobenzyl)pyridine (NBP) 122 as an analytical reagent for the estimation of the concentrations of specific alkylating agents was first proposed by Epstein *et al.*[100] According to this procedure, a mixture of the alkylating agent 123 and NBP 122 is heated

for a standard period of time (20 min). After cooling and introduction of alkali the intensity of color developed due to the formation of the product 124 is measured spectrophotometrically at 600 nm. Absolute concentrations are determined using a standard curve.



Weinstein *et al.*[48] using ^{14}C-CCNU labelled in the 2-chloroethyl portion of the molecule reported reactions with poly U, poly A, poly G, poly C, tRNA, DNA and protein which resulted in bound ^{14}C. They also observed[48] ^{14}C binding to tRNA, DNA and protein of leukemia L 1210 cells *in vitro*. Similar results were reported by Connors and Hare[101] for the macromolecules of murine TLX5 cells following administration of ^{14}C-CCNU to innoculated mice. Ludlum *et al.*[81] reported that the relative extent of binding of ^{14}C-BCNU, labelled in the 2-chloroethyl portion of the molecule, to synthetic polynucleotides was poly C >> poly G > poly A, poly U. Kramer, Fenselau and Ludlum,[81,102] upon incubation of BCNU 5 with poly C and subsequent hydrolysis of the polymer, isolated two products which they identified as 3-(2-hydroxyethyl)-cytidine monophosphate 125 and 3,N^4-ethanocytidine monophosphate 126. They suggested[102] that a 3-(2-chloroethyl)cytidine 127 moiety may be an intermediate in the formation of both products. A similar experiment[102] involving poly G resulted in the isolation of 7-(2-hydroxyethyl)guanine monophosphate 128.



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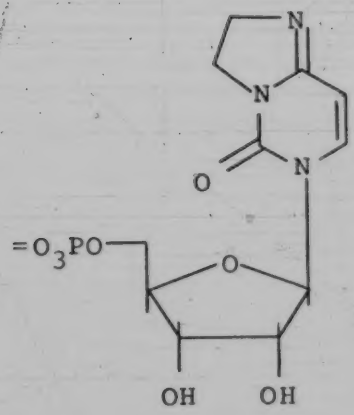

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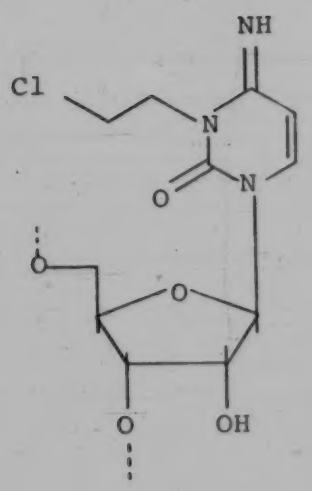

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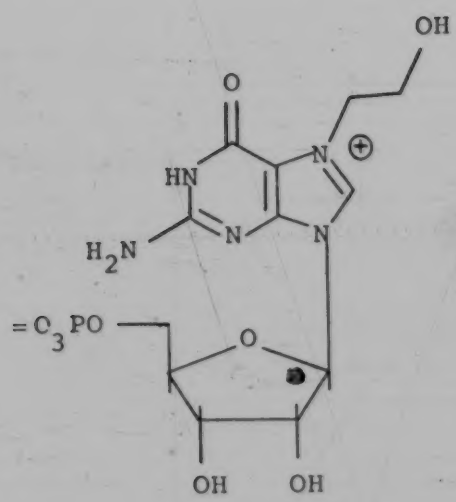

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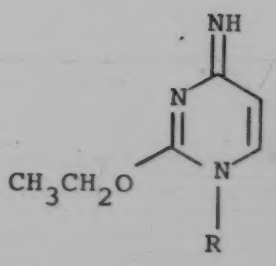
While extensive studies with mono- and polynucleotides[92] have indicated that the 3-position of cytidine is the most readily alkylated, recent research[103] suggests that alkylation can also occur at the N^4 and O^2 positions of cytidine. Singer[103] reacted ENU 10 with cytidine in aqueous solution at pH 7.3. Of the products isolated 50% contained the ethyl group bound to the O^2 position 129, 31% was N^4-ethylcytidine 130 and 19% was 3-ethylcytidine 131. At pH 6.1 the products were isolated in 52%, 36% and 13% yields, respectively.



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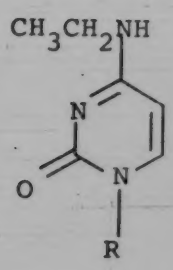

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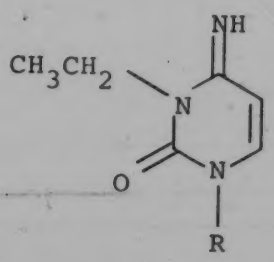

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In addition to alkylation, a number of 2-haloethyl-nitrosoureas produce DNA interstrand cross-links.

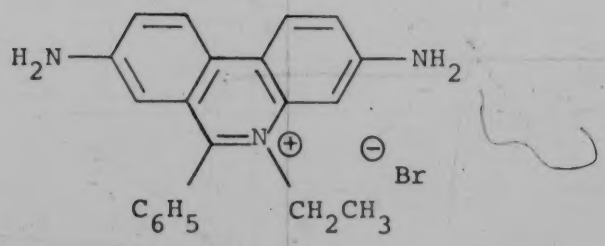
Kohn[104,105] has reported the formation of cross-links *in vitro* and more recently[106] *in vivo* after exposure to BCNU 5. Many bifunctional alkylating agents have been reported[107-111] to produce DNA interstrand cross-links. Bifunctional alkylating agents are generally more cytotoxic than monofunctional derivatives.[112]

The major objective of this portion of the present study was to elucidate the mechanism by which some nitrosoureas produce significant DNA interstrand cross-linking, since they are not obviously bifunctional alkylating agents.

Studies Related to the Alkylation of DNA by Nitrosoureas

DNA alkylation by nitrosoureas was measured by two methods. Relative abilities to alkylate PM2-covalently-closed-circular-DNA (PM2-CCC-DNA) was examined using the rapid and convenient ethidium fluorescence assay. The absolute extent of alkylation was measured for one 2-chloroethylnitrosourea (CCNU 6) at different concentrations using radiolabelling techniques.

Ethidium bromide 132 is a trypanocidal dye that interacts with DNA. Le Pecq and Paoletti[113] as well as Morgan and Paetkau[114] have observed a marked increase in the fluorescence of the dye in the presence of bihelical nucleic acids while no enhancement is observed in the presence of single stranded nucleic acids. Le Pecq and



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and Paoletti[113] concluded that the ethidium cation binds to duplex regions of nucleic acids by intercalation between base planes. Their results suggested that ethidium bromide binds once for every five nucleotides, a suggestion consistent with previous X-ray diffraction data.[115] They proposed that the fluorescence enhancement is due to the occlusion of the ethidium cation, by intercalation, into the hydrophobic region of the nucleic acids where it is protected against quenching by the aqueous solvent. Additional experiments[113] supporting this hypothesis indicated that the fluorescence of ethidium bromide increases when it is measured in alcohols of decreasing hydrophilic character.

Morgan and Paetkau observed,[114] that when an ethidium bromide concentration of 0.5 μg/ml was employed, a linear response of fluorescence with bihelical DNA concentration up to 0.02 A_{260} was obtained. The observation that fluorescence is directly proportional to the amount of double stranded DNA in solution has permitted the

development of a convenient assay for measuring alkylation of DNA.

Alkylation is detected with PM2-CCC-DNA. Using the ethidium fluorescence assay, aliquots of a reaction mixture containing DNA are analyzed for base alkylation by dilution with a solution of ethidium bromide buffered to pH 11.8. The fluorescence of the DNA-ethidium solution is measured to obtain an estimate of the total DNA concentration. The resultant solution is then heat denatured (96°C/3min) and cooled quickly (0°C). Under these conditions native PM2-CCC-DNA returns to register, thus the fluorescence after the heating-cooling cycle is the same as that obtained initially. Alkylated PM2-CCC-DNA undergoes a facile depurination or depyrimidination in the reaction mixture or during the heat denaturation to produce apurinic sites which hydrolyze quickly in the hot alkaline solution. The resulting open circular DNA(OC-DNA) heat denatures to form one circular strand and one linear strand which do not bind ethidium bromide and the fluorescence falls to zero. By observing the decrease in fluorescence, after the heating-cooling cycle, of aliquots taken from the reaction mixture, the relative extent of DNA alkylation can be monitored. The assay is illustrated in Figure 13.

Alkylation measured with the ethidium bromide fluorescence assay was observed for all of the nitrosoureas

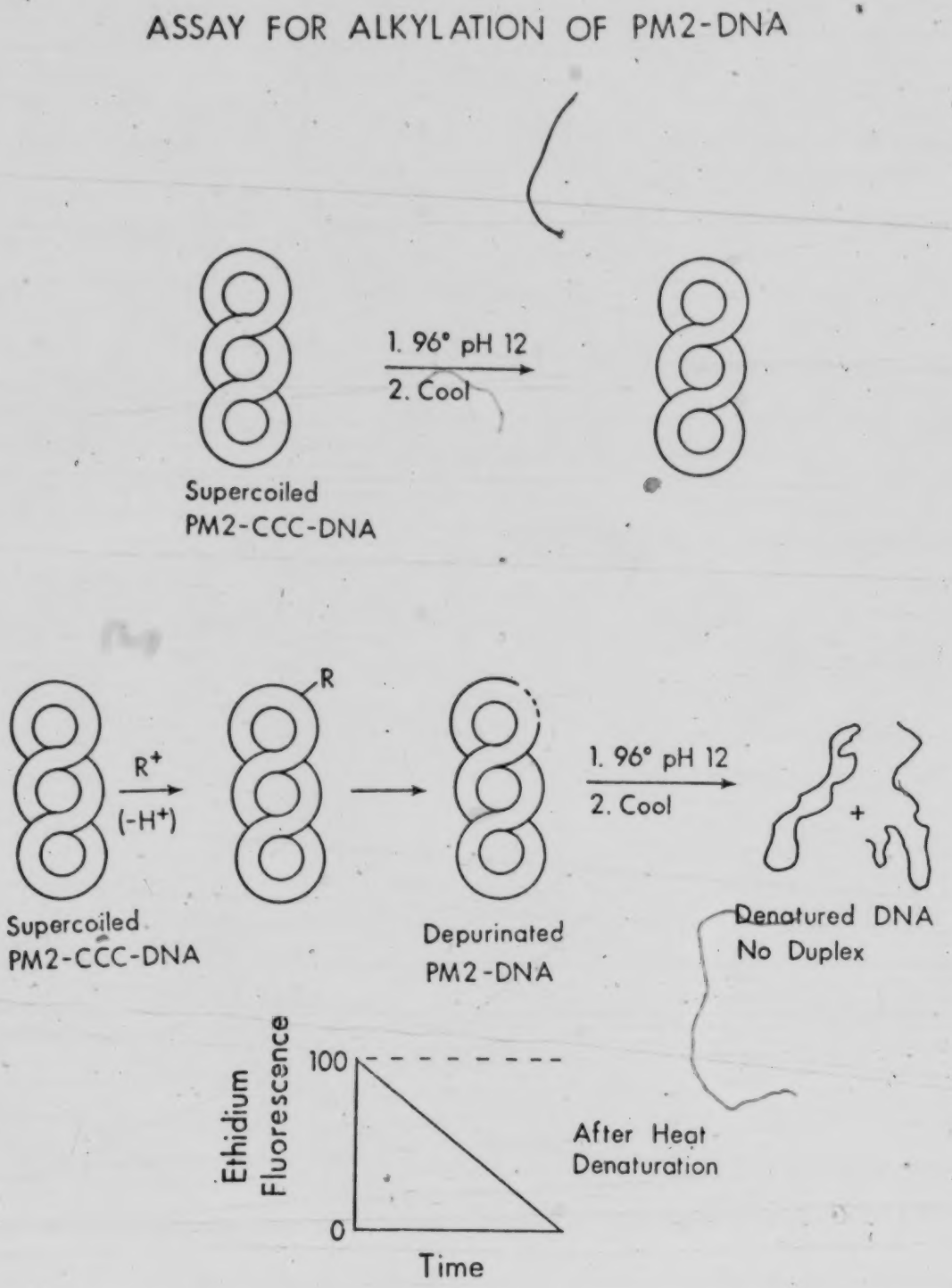

Figure 13. Ethidium bromide assay for DNA base alkylation of PM2-DNA.

prepared in this study. Typical examples of alkylation curves are shown in Figure 14 for a series of 1,3-bis(2-haloethyl)-1-nitrosoureas. The different behavior exemplified by BCNU 5 results from the occurrence of more extensive DNA interstrand cross-links after initial alkylation which prevents heat denaturation of the PM2-OC-DNA (see following section).

In addition to the effects of cross-linking, spectrofluorometric measurement of DNA alkylation can also be complicated by concomitant single strand scission which results in a similar decrease in fluorescence after the heating-cooling cycle. Additionally, while the assay can measure the rate of initial alkylation of the DNA molecule it does not allow measurement of the extent of alkylation since, in principle, one alkylation per molecule is sufficient for the depurination, alkaline strand scission and heat denaturation to occur. Therefore, DNA alkylation was also measured directly using radiolabelling techniques.

λ-DNA was treated with ^{14}C-CCNU labelled in the 2-chloroethyl portion of the molecule. After a reaction time of 6 hours in a pH 7.2 buffered solution at 37°C, unbound drug was removed from the reaction mixture by dialysis. DNA alkylation was then measured by liquid scintillation counting to determine bound drug concentration and ultraviolet absorbance was used to determine

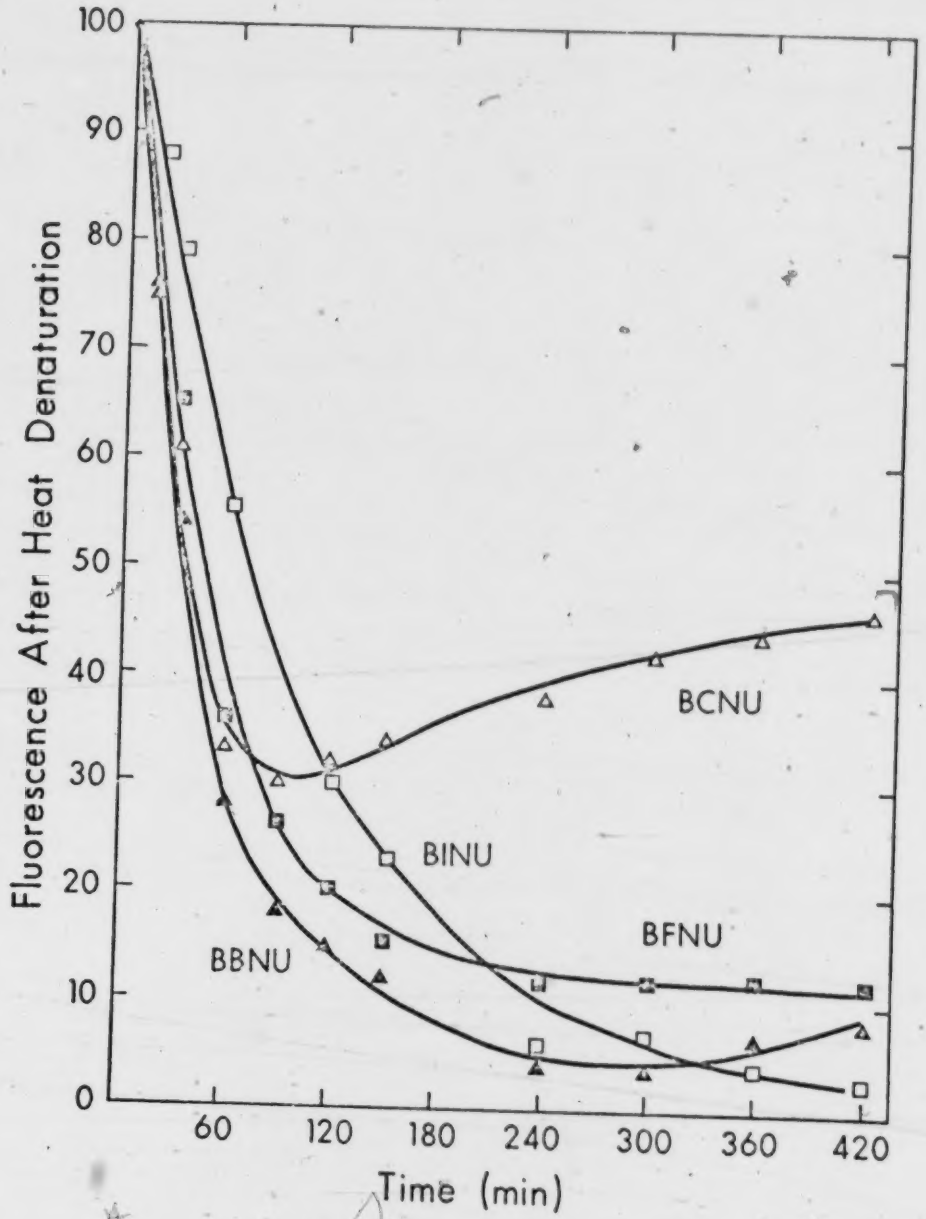

Figure 14. Alkylation of PM2-CCC-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM: (△) BCNU 5; (▣) BFNU 26; (▲) BBNU 62; or (□) BINU 63.

DNA concentration. The results from the present study and those from a previous one[116] involving mitomycin C 133 are reported in Table 12. As can be observed from

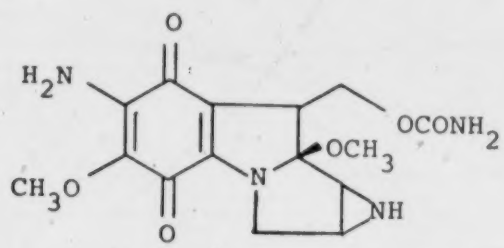

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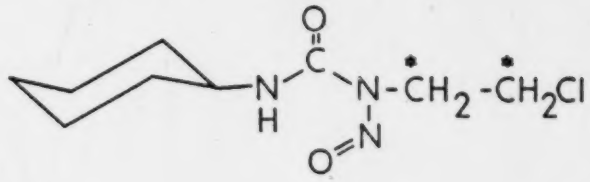
Table 12, even at twice the concentration, the extent of alkylation by CCNU 6 was much lower than that observed for mitomycin C 133. Additionally, the amount of nitrosourea which hydrolyzed was far greater than in the case of mitomycin C 133. Both of these observations suggest that CCNU 6 is much less selective in its reaction with nucleophiles, even reacting to a large extent with water, and imply that alkylation by nitrosoureas is largely an S_N1 or a low activation S_N2 process.

DNA Interstrand Cross-Linking by Nitrosoureas.

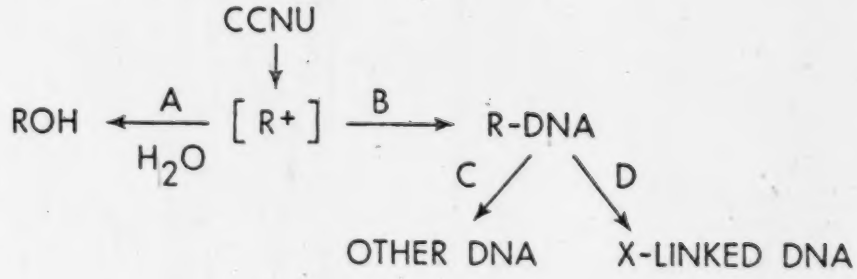
The ethidium fluorescence assay allows detection and estimation of the amount of covalently linked complementary DNA, (CLC-DNA) produced after reaction with

Table 12

BINDING OF 14-C-LABELLED CCNU TO DNA



	CCNU			MITOMYCIN C
Drug Concn. (M)	2.19×10^{-4}	1.03×10^{-3}	6.5×10^{-3}	1.2×10^{-4}
Maximum % Cross-Linked DNA	5 ± 1	39 ± 1	47 ± 1	84 ± 2
Nucleotide Equivalents / Bound Drug	2131 ± 74	484 ± 12[a]	73 ± 7	50 ± 5
Hydrolyzed Drug (A/B) / Bound Drug	3271 ± 148	3498 ± 21	3331 ± 335	42 ± 4

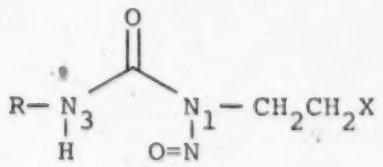


a (C/D) = 430

an appropriate drug.[116,117] Using the assay, aliquots of cross-linked DNA were analyzed for CLC sequences by dilution with a solution of ethidium bromide buffered to pH 11.8. The fluorescence of the DNA-ethidium solution was measured to obtain an estimate of the total DNA concentration. The solution was then heat denatured (96°/3 min), cooled quickly (0°C) and the fluorescence of the solution was again measured. Under these conditions separated DNA strands do not reanneal. CLC-sequences, by virtue of the chemical cross-link which acts as a nucleation point, reanneal and result in double stranded DNA which binds ethidium bromide. The ratio of the fluorescence after heating to the fluorescence before heat denaturation was then a measure of the extent of interstrand cross-linked DNA. The assay was conducted at pH 11.8 to prevent spontaneous formation of short intrastrand duplex structures resulting from accidental self-complementarity after heating and cooling. At pH 11.8 such structures are unstable when compared with those formed by CLC-DNA.[114,118] That this assay procedure detected the formation of CLC-DNA as a result of a chemical cross-linking event has been confirmed by experiments with the enzyme S_1-endonuclease.[116] This enzyme specifically cleaves single-stranded DNA and is essentially inactive on duplex DNA. Therefore, it distinguishes DNA which is renaturable because of a chemical cross-link

from DNA which separates into single-strands upon heating. The cross-linking assay is illustrated in Figure 15.

Bis(2-haloethyl)nitrosoureas 26, 5, 62, 63 cross-link λ-DNA under physiological conditions [e.g., at pH 7.2 and 37°C, BCNU 5 cross-links 42% of λ-DNA in 6 h (Fig. 16)]. At 50°C a significant increase in the rate but not the extent of DNA interstrand cross-links was observed (e.g., at pH 7.2 and 50°C, BCNU 5 cross-links 37% of λ-DNA in 2 h). Interstrand cross-linking was most efficient for compounds containing the 2-chloroethyl function and in such cases the rate was observed to increase with increasing pH (Fig. 17) in accord with suggested mechanisms of decomposition to produce alkylating species (see Chapter II). The cross-links were observed to be stable for at least 48 h in 0.15 M NaCl and 0.015 M sodium citrate, conditions which have been reported[119,120] to reverse the interstrand cross-links produced by carzinophillin, which suggests that the nitrosoureas produce two covalent bonds. The extent of cross-linking is unrelated to the nature of the urea 134 N-3 substitutents, provided the nitrogen carries at least one hydrogen.



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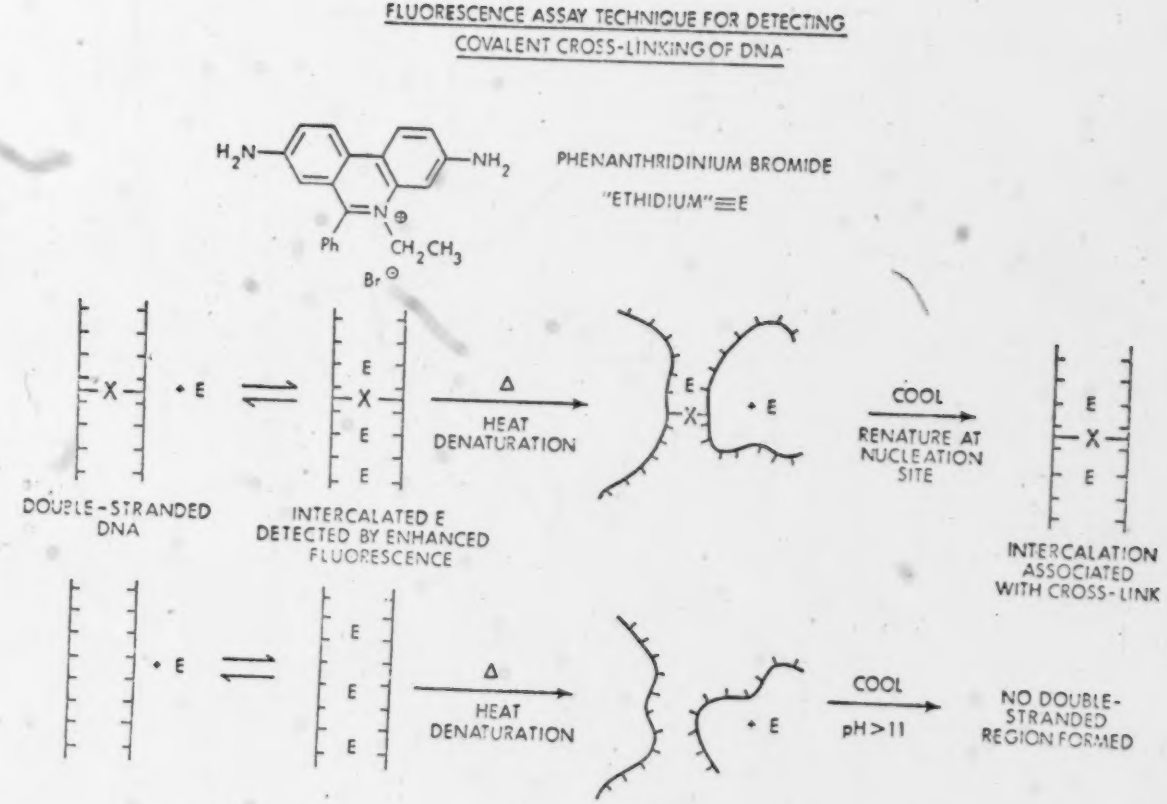


Figure 15. Ethidium bromide assay for the detection of DNA interstrand cross-linking of λ-DNA.

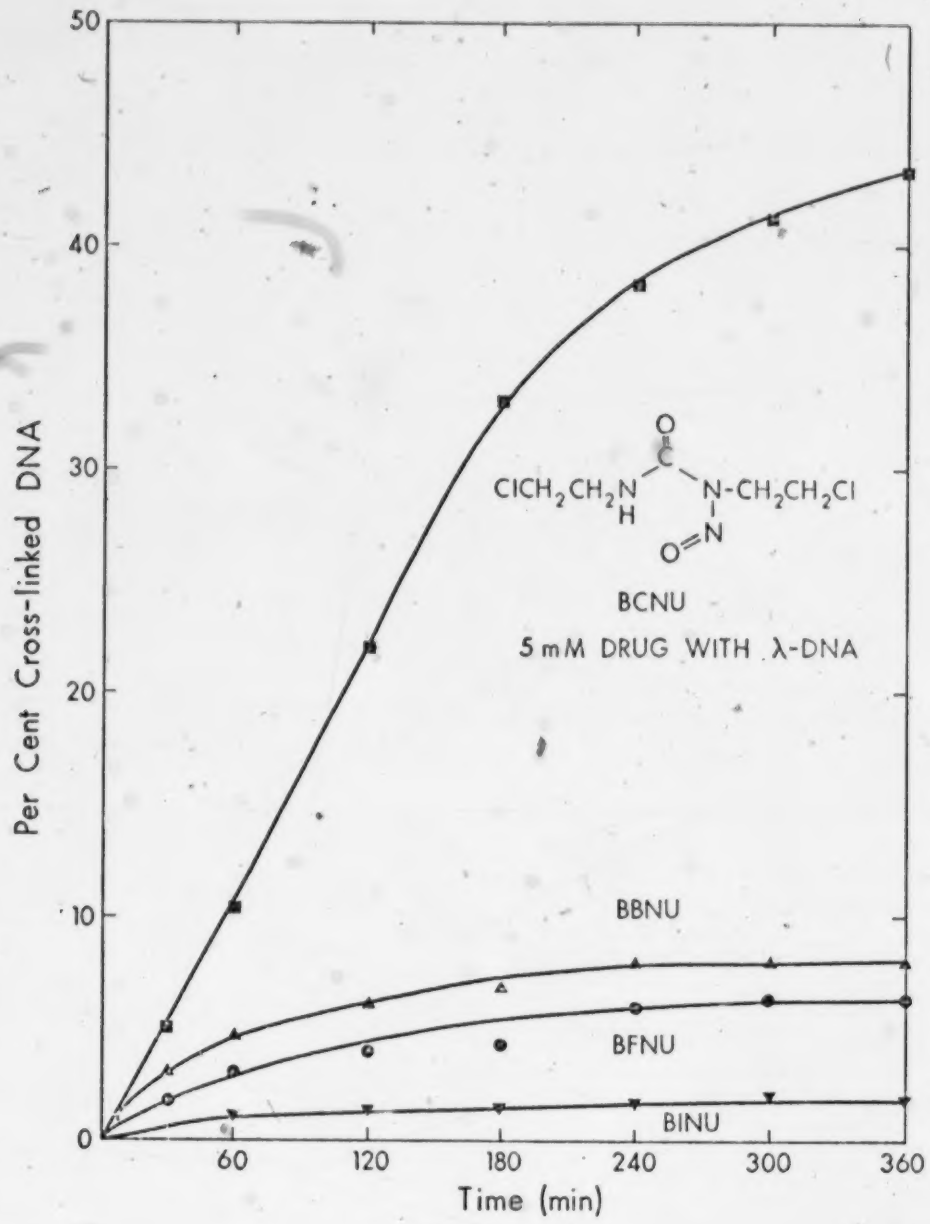


Figure 16. DNA interstrand cross-linking of λ-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM: (■) BCNU 5; (▲) BBNU 62; (●) BFNU 26; or (▼) BINU 63.

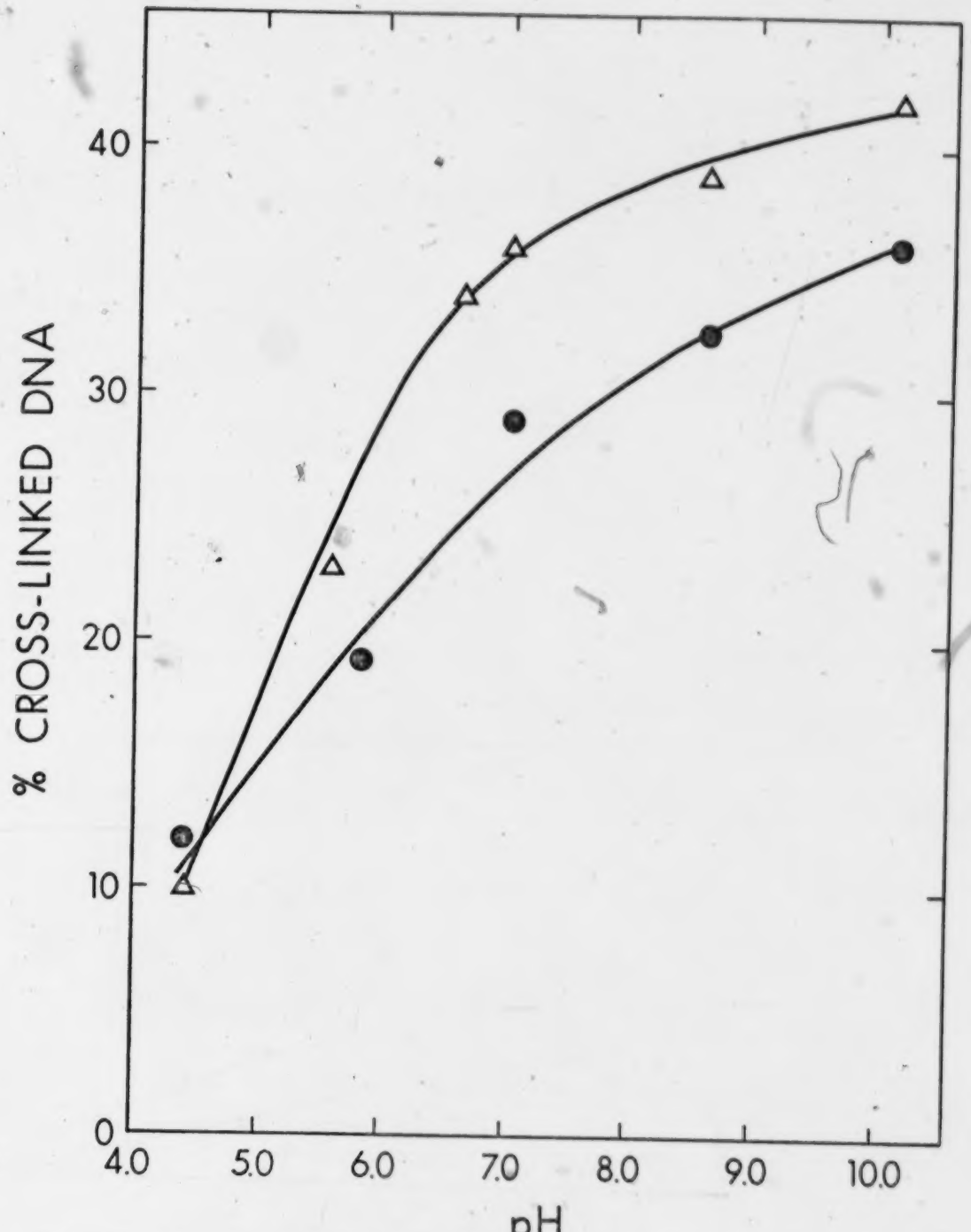
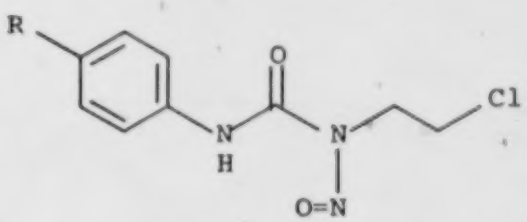


Figure 17. pH dependence of DNA cross-linking. Reaction of λ-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 50°C with 5 mM: (●) BCNU 5 or (Δ) chlorozotocin 86. Cross-linking measured after a reaction time of 60 min.

Aryl N-3 substitution substantially increases the rate but not the extent of cross-linking by increasing the acidity of the N-3 proton in accord with the results of the pH study. Further attempts to affect the acidity of the N-3 proton were accomplished by preparing 1-(2-chloroethyl)-3-p-nitrophenyl-1-nitrosourea 69 and 1-(2-chloroethyl)-3-p-methoxyphenyl-1-nitrosourea 68. However, neither derivative exhibited a cross-linking rate which differed significantly from the unsubstituted 1-(2-chloroethyl)-3-phenyl-1-nitrosourea 135.

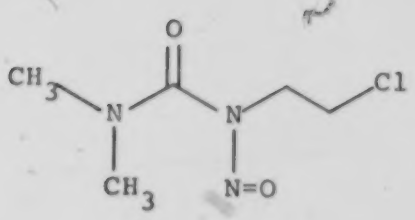


68 R = OCH_3

135 R = H

69 R = NO_2

As expected, no interstrand cross-linking was observed for 1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea 70.



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These initial observations are in accord with research outlined in the introduction of this chapter which indicates that the 2-haloethyl function is primarily responsible for the reactions with DNA.

The extent of DNA interstrand cross-linking by 2-chloroethyl nitrosoureas was observed to increase with the guanosine plus cytidine (G + C) content of natural DNAs (Fig. 18). The average number of cross-links per nucleotide produced by BCNU 5 and CCNU 6 was calculated using a Poisson's distribution[110] of the cross-links and assuming that one link per molecule is sufficient to permit spontaneous renaturation. The values obtained for *Clostridium perfringens* DNA (30% G + C), calfthymus DNA (40% G + C) and *E. coli* DNA (50% G + C) has been calculated as 1.4×10^{-5}, 4.2×10^{-5} and 9.1×10^{-5} cross-links per nucleotide, respectively. Similar results of 1.1×10^{-5}, 2.8×10^{-5} and 7.7×10^{-5} cross-links per nucleotide were obtained for CCNU for DNAs of 30%, 40% and 50% (G + C) content, respectively (Fig. 18). These values neglect the effects of strand breakage.

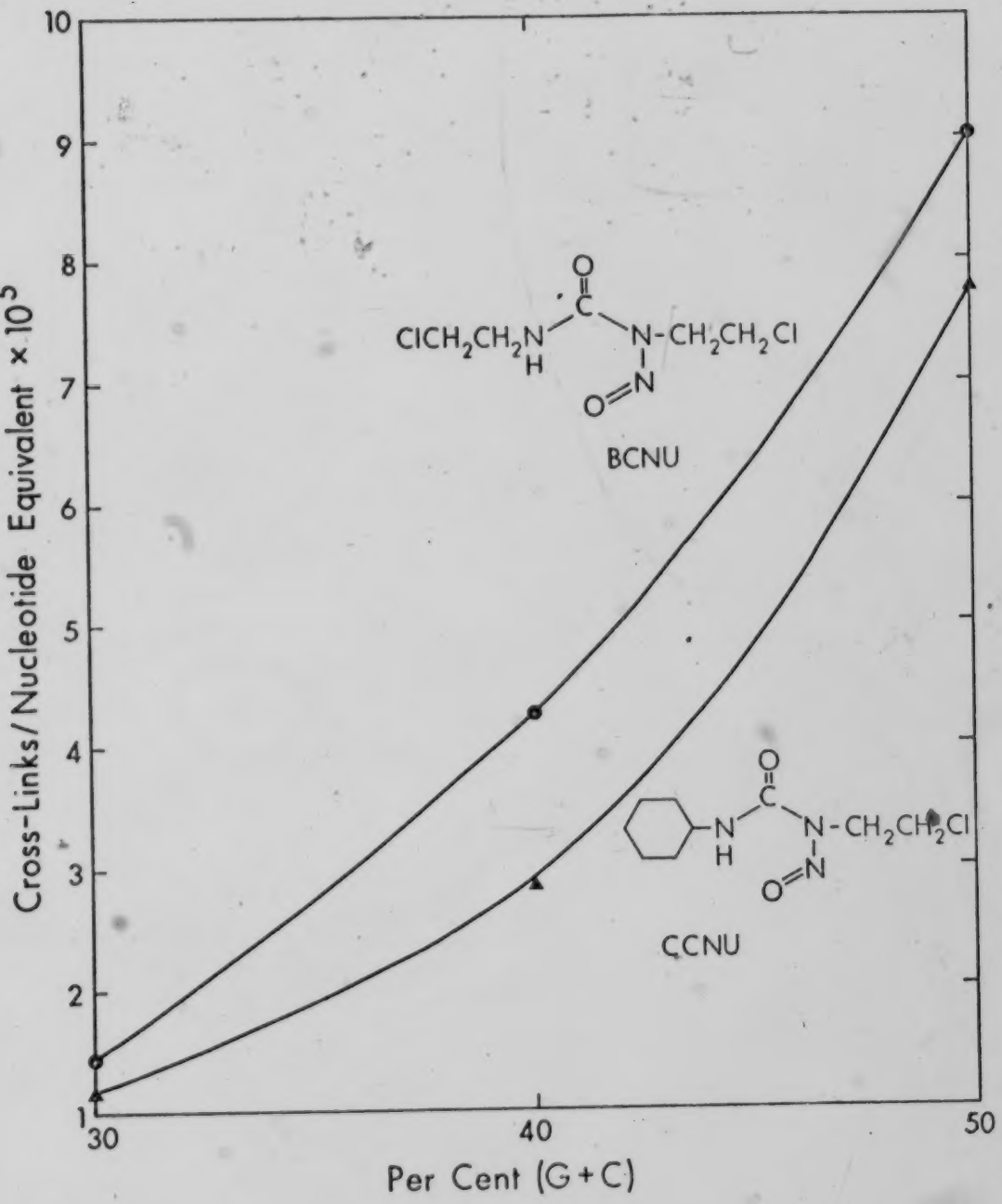
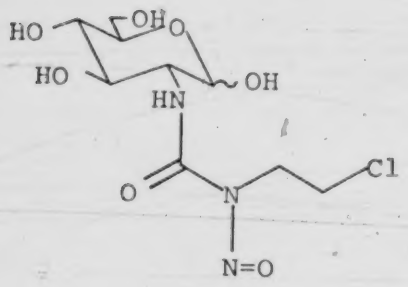


Figure 18. Dependence of DNA interstrand cross-linking on on G + C content of DNA. *C. perfringens* (30% G +C), calf thymus (40% G + C), or *E. coli* (50% G + C) 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM: (●) BCNU 5 or (▲) CCNU 6.

Cross-linking with bis(2-haloethyl)nitrosoureas showed a strong halogen dependence: Cl, 42%; Br, 8%; F, 7%; I, <2% (Fig. 16). However, only one 2-haloethyl group was necessary for cross-linking, in accord with the proposed decomposition to give 2-haloethyl alkylating agents (see Chapter II). For example, the antitumor agent chlorozotocin 86 cross-links DNA very efficiently, whereas the related streptozotocin 87, lacking the 2-haloethyl function, does not.



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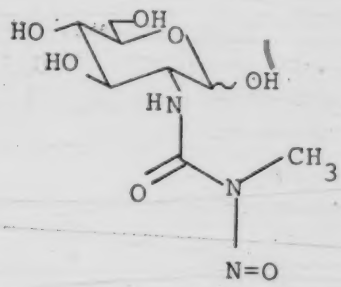

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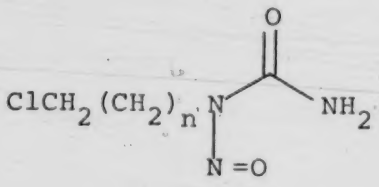
Cross-linking is not observed when the halogen is replaced by -OH or $-OCH_3$ which suggests that the ability of the halogens to act as leaving groups may be related to the cross-linking mechanism. This is in agreement with a suggestion by Ludlum[81,102] that a 2-chloroethyl cytidine moiety might retain alkylating activity and allow additional DNA modification. Kohn has also suggested[105] that

substitution of the chlorine atom by a second nucleophilic site in the DNA molecule after initial chloroethylation could lead to the observed DNA interstrand cross-links.

Steric effects as they relate to DNA cross-linking by 2-chloroethylnitrosoureas were also investigated. Neither 1,3-bis[1-(chloromethyl)ethyl]-1-nitrosourea 61 (BCNU-α-Me) (Fig. 7) nor 1,3-bis(2-chloropropyl)-1-nitrosourea (BCNU-β-Me) 60 (Fig. 7) exhibited any ability to produce DNA interstrand cross-links.

In addition to the halogen dependence and steric effects, the position of the halogen was also observed to affect cross-linking ability. Four nitrosoureas 3, 57, 58, and 59 were prepared. While all of these



3 CNU n = 1

57 CPNU n = 2

58 4-CBNU n = 3

59 5-CPNU n = 4

derivatives alkylate DNA significantly, only the 2-chloroethyl derivative produced covalent interstrand cross-links (CNU cross-links 36% of λ-DNA in 6 h).

The observation that alkylation by 2-chloroethylnitrosoureas was extensive within the first two hours (Fig. 14) and yet the extent of cross-linking did not reach a maximum for nearly six hours (Fig. 16) suggested that two distinct steps were involved in the mechanism. Two compounds, BCNU 5 and CCNU 6 were incubated with λ-DNA for two hours at which time the extent of interstrand cross-linking was observed to be 15% and 17%, respectively. The reaction mixtures were then quenched at 0°C and dialyzed for 15 h at 4°C to remove unreacted drug. Upon incubation of the dialysate at 37°C initial readings indicated that the percentage of cross-linked DNA had not changed significantly during the dialysis. Subsequent readings showed that the extent of cross-linking continued to increase for the next 4.5 hours and resulted in over 35% cross-linked DNA at that time (Fig. 19). If the incubation temperature was raised to 50°C a corresponding increase in the rate of cross-linking after dialysis was observed. A control experiment was run in parallel to show that all the free nitrosourea could be removed by dialysis (see experimental). This suggests that the initial alkylation (on guanosine or cytidine) is of a low activation energy and that the

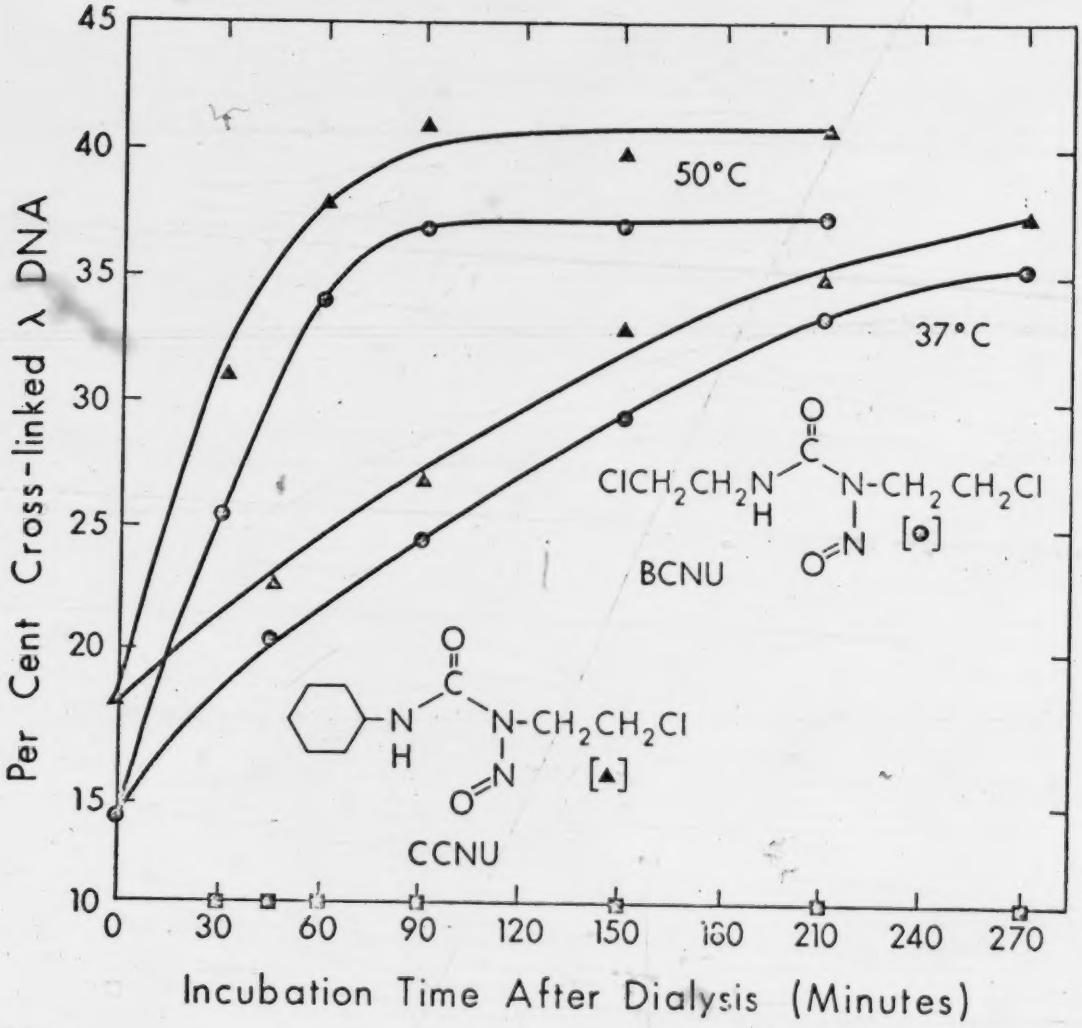


Figure 19. Incubation of λ-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM: (●) BCNU 5 or (▲) CCNU 6 for 2 h. Samples were dialyzed 15 h at 40°C in 0.05 M phosphate buffer pH 7.2 and followed by reincubation at 37°C or 50°C. (■) control, 5 mM BCNU 5 added to λ-DNA 1.0 A_{260} just prior to dialysis followed by reincubation as described above.

second bond is formed by a slower alkylation of higher activation energy.

This observation could be significant with respect to the antitumor activity of 2-chloroethylnitrosoureas. Therefore, a comparison between DNA interstrand cross-linking and activity against leukemia L1210 is shown in Table 13.

It is evident that only compounds containing the 2-fluoroethyl- or 2-chloroethyl- moiety exhibit significant activity. An observation in accord with the decomposition studies reported in Chapter II which indicated that only 2-fluoroethyl- and 2-chloroethylnitrosoureas produce significant amounts of the haloethyl alkylating agent. The loss of cross-linking ability by chain lengthening or chain branching in the 2-chloroethyl analogues parallels the structure activity studies of Montgomery[3] (Table 13) who observed that similar structure modifications resulted in low activity or loss of activity against the L 1210 test system. The correlation between extent of DNA cross-linking and antileukemic activity observed in Table 13 initiated a more detailed study of the molecular mechanisms involved in the cross-linking reactions.

Previous research by Ludlum *et al.*[102] has indicated that the cytidine residues are alkylated most extensively by chloroethylnitrosoureas. In accord with this result is the observed dependence of the extent of

Table 13

Covalent Cross Linking of λ-DNA by 2-Haloethylnitrosoureas and Correlation with Activity against Leukemia L1210

#	R	R'	DNA Cross-Linking(%)[a]	Activity against Leukemia L1210[b] I_p (10^6cells) log kill[c]	% cures	Ref.
2	H-	$-CH_3$	0	-	10	121
10	H-	$-CH_2CH_3$	0	Inactive		122
3	H-	$-CH_2CH_2Cl$	36	5	40	
61	$ClCH_2CH(CH_3)-$	$-CH(CH_3)CH_2Cl$	0	-	-	
115	$ClCH_2CH(Et)$	$-CH(Et)CH_2Cl$[d]	-	Inactive	-	
60	$ClCH(CH_3)CH_2-$	$-CH_2CH(CH_3)Cl$	0	5	20	
136	cyclo-$C_6H_{11}-$	$-CH_2CH(CH_3)Cl$[d]	-	5	0	
57	H-	$-CH_2CH_2CH_2Cl$	0	-	-	
137	C_6H_5-	$-CH_2CH_2CH_2Cl$[d]	-	2	0	
26	FCH_2CH_2-	$-CH_2CH_2F$	6	6	50-60	
5	$ClCH_2CH_2-$	$-CH_2CH_2Cl$	37	6	0-100	
62	$BrCH_2CH_2-$	$-CH_2CH_2Br$	7	5	20	
63	ICH_2CH_2-	$-CH_2CH_2I$	<2	Inactive	0	

continued....

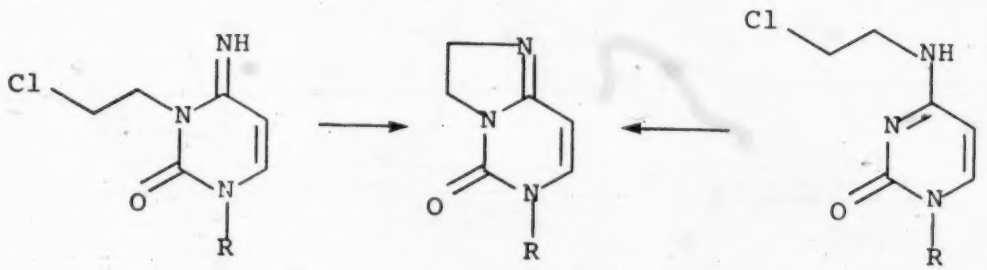
Table 13 (continued)

64	cyclo-C_6H_{11}	$-CH_2CH_2F$	6	6	70-90	
6	cyclo-C_6H_{11}	$-CH_2CH_2Cl$	37	5	80-100	
65	cyclo-C_6H_{11}	$-CH_2CH_2Br$	8	6	30	
66	cyclo-C_6H_{11}	$-CH_2CH_2OH$	0	-	-	
67	cyclo-C_6H_{11}	$-CH_2CH_2OCH_3$	0	-	-	
138	*cis*-2-Cl-cyclo-C_6H_{11}	$-CH_2CH_2Cl$	41	-	30-90	
139	*trans*-2-Cl-cyclo-C_6H_{11}	$-CH_2CH_2Cl$	41	-	80-100	
135	phenyl	$-CH_2CH_2Cl$	36	3	0	
69	p-nitrophenyl	$-CH_2CH_2Cl$	37	-	-	
68	p-methoxyphenyl	$-CH_2CH_2Cl$	37	5	0	
140	2-adamantyl	$-CH_2CH_2Cl$	42		≥50	123
87		Streptozotocin	0		Inactive[e]	124
86		Chlorozotocin	41	6	90	124
70		$(CH_3)_2NC(=O)N(NO)CH_2CH_2Cl$	0		Inactive	11

[a] 50° pH 7.2 after 2 hr of reaction. [b] Antileukemic data from reference[3] unless otherwise stated for single does Ip drug injection. [c] Reduction of an inoculum of 10^5 cells to 10^2 cells is a 3 log kill.[1] [d] These compounds were not assayed for DNA cross-linking in this study, however activity is shown for comparison of 2-chloropropyl and 3-chloropropyl derivatives. [e] Shows activity on a qd 1-9 schedule [J.M. Venditti, Cancer Chemotherap. Rep., 2, 35 (1971)].

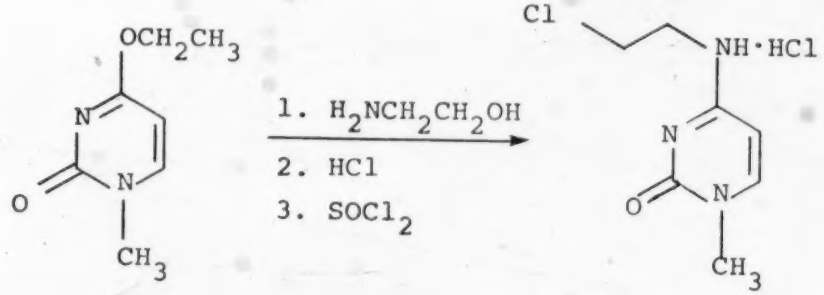
cross-linking on the (G + C) content of natural DNAs (Fig. 18). The two most likely positions of cytidine for chloroethylation to occur appear to be the 3 and the N^4 positions.

The 3 position of cytidine residues in helical DNA has been observed to be alkylated both *in vitro* and *in vivo*.[92] Treatment of DNAs from a number of sources with alkylating agents such as MNNG 1, MNU 2 or ENU 10 has resulted in the isolation of small amounts of 3 alkylated cytidine.[92] Chloroethylation at the 3 position of cytidine residues might produce an intermediate which would retain alkylating activity as suggested by Ludlum.[81,102] Chloroethylation of the N^4 position results in a nitrogen half-mustard which appears to be the most likely chloroethyl cytidine derivative which could retain alkylating activity. As described previously, Ludlum *et al.*[81,102] isolated 3,N^4-ethanocytidine monophosphate 126 after treatment of poly C with BCNU 5. Transfer of a 2-chloroethyl alkylating agent to either the 3 position 127 or N^4 position 141 of cytidine could account for the production of this simple intramolecular bifunctional alkylation product.



127 126 141

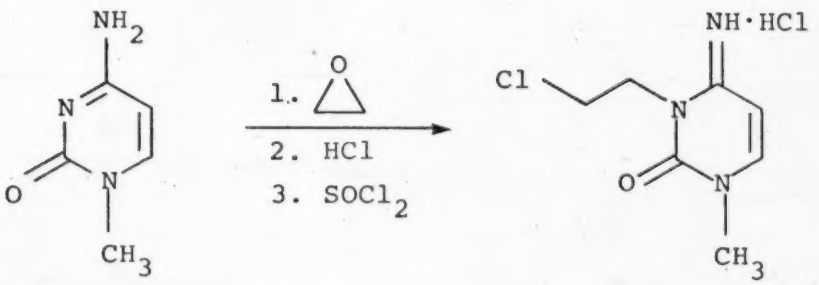
Two model compounds were prepared to test the alkylating activity of chloroethylcytidine intermediates. N^4-(2-chloroethyl)-1-methylcytosine hydrochloride 142 was prepared according to the method of Ueda and Fox,[125] by reaction of 4-ethoxy-1-methyl-2-pyrimidone 143 with 2-aminoethanol followed by chlorination with thionyl chloride. 3-(2-chloroethyl)-1-methylcytosine hydrochloride



143 142

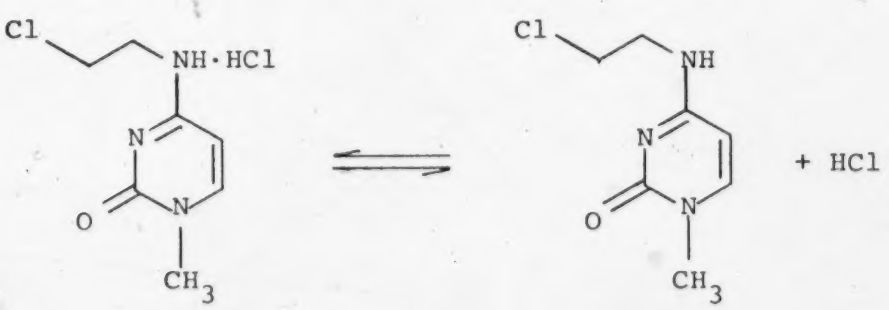
144 was prepared by the action of ethylene oxide on 1-methylcytosine 145 followed by chlorination with thionyl

chloride.



145 144

In D_2O the latter compound exhibits an uncomplicated pmr spectrum. However, the former compound in D_2O produces a pmr spectrum containing two sets of resonances. Heating induces coalescence of the signals suggesting a tautomeric or acid base equilibrium. When the D_2O solution is buffered to pH 7.2 only one form is present. A u.v. spectrum indicates the presence of only one compound (λ_{max} 276 nm). These results suggest that the two sets of signals observed in D_2O result from the presence of the free base



142 146

146 and the protonated form 142, while in buffered solution only 146 is present.

As observed in Figure 20, both chloroethylcytosine derivatives exhibit significant levels of alkylation of PM2-CCC-DNA. This suggests that similar derivatives produced after chloroethylation of the DNA polymer would retain intermolecular alkylating activity and result in DNA interstrand and intrastrand cross-links. Two processes could account for the alkylating activity of these derivatives (i) a simple bimolecular S_N2 nucleophilic substitution of the chlorine or (ii) labilization of the carbon-chlorine bond to produce a carbonium ion or similar activated intermediate by an S_N1 process followed by a rapid reaction with nucleophiles.

If the first process is operative then other chloroalkylcytidine derivatives in which the chlorine is bonded to a primary carbon atom should undergo further alkylation and produce interstrand cross-links. As previously described, the 3-chloropropyl-, 4-chlorobutyl- and 5-chloropentyl-nitrosoureas exhibit no cross-linking ability.

The relative alkylation of DNA in physiologically buffered solution at 37°C for these three nitrosoureas are 81, 82 and 73, respectively. Decomposition of 1-(3-chloropropyl)-1-nitrosourea (CPNU) 57 in aqueous solution

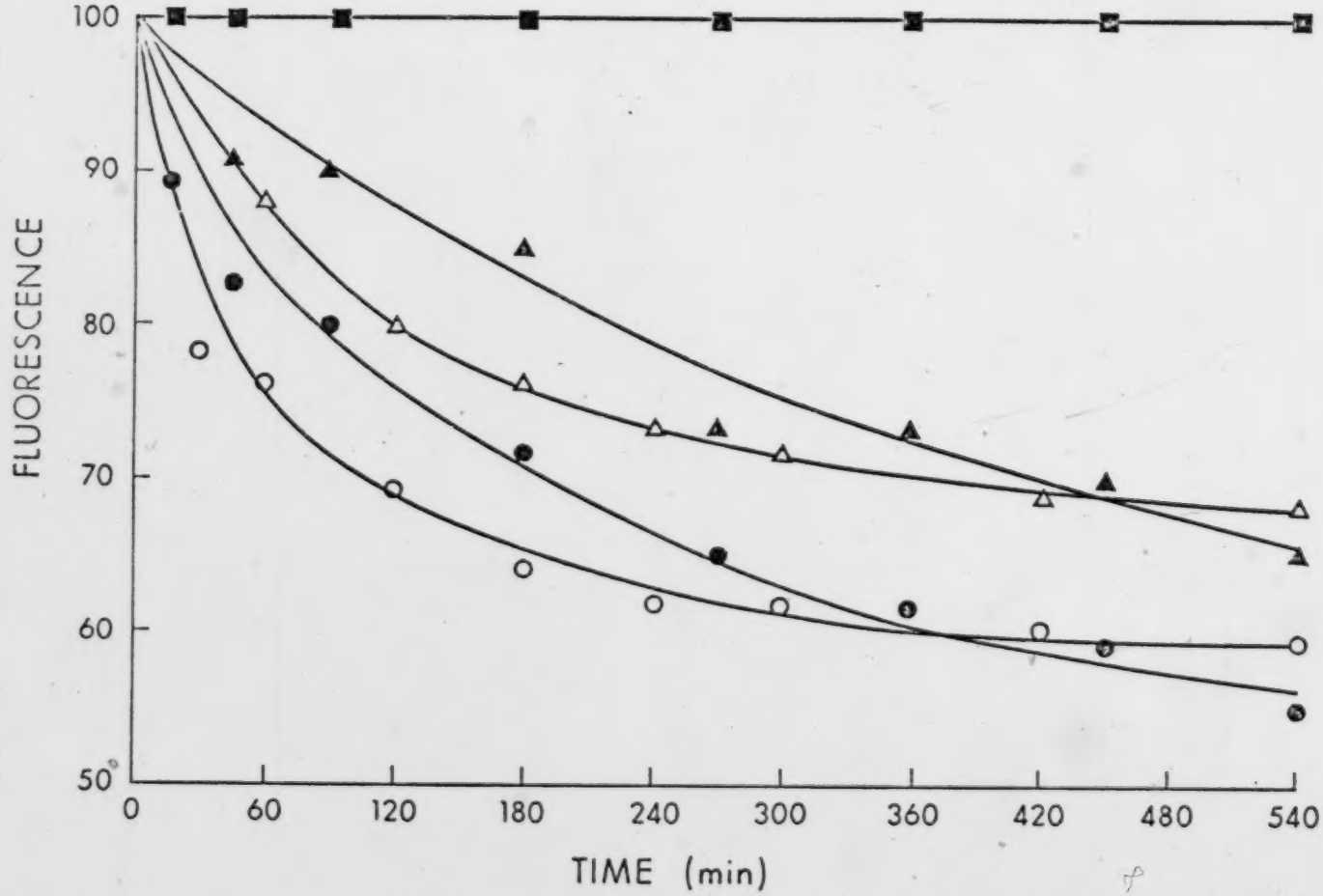


Figure 20. Reaction of PM2-CCC-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with N^4-(2-chloroethyl)-1-methylcytosine hydrochloride 142: (▲) 5 mM or (●) 10 mM; or 3-(2-chloroethyl)-1-methylcytosine hydrochloride 144: (Δ) 5 mM or (O) 10 mM.

$ClCH_2CH_2CH_2N(NO)CONH_2$

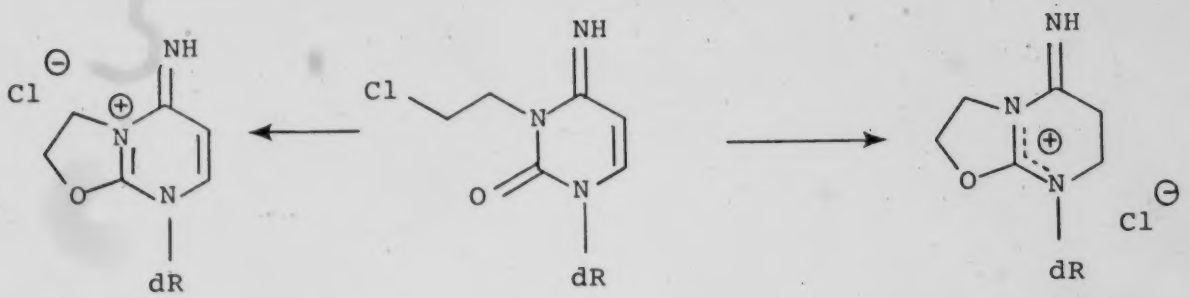
57

buffered to pH 7.2 at 37°C for 24 h was followed by gas-liquid chromatography. The observation that 3-chloropropanol accounted for 95% of the volatile products indicates the extensive formation of a 3-chloropropyl alkylating agent. Presumably 3-chloropropylated nucleosides result when DNA is treated with CPNU 57 but these intermediates do not result in cross-links. Since only chloroethylated nucleosides appear to result in interstrand cross-links labilization of the carbon-chlorine bond appears to be a prerequisite for cross-linking activity.

N^4-(2-chloroethyl)cytidine 141 is essentially an aromatic nitrogen half-mustard derivative. While there is disagreement[125-129] concerning the mechanism by which aromatic nitrogen mustards undergo alkylations, it is generally agreed that the mechanism resembles an S_N1 nucleophilic substitution. Nitrogen mustard alkylation could explain the ability of N^4-chloroethylcytidine 141 residues to produce DNA cross-links while other N^4-chloroalkylcytidine residues do not.

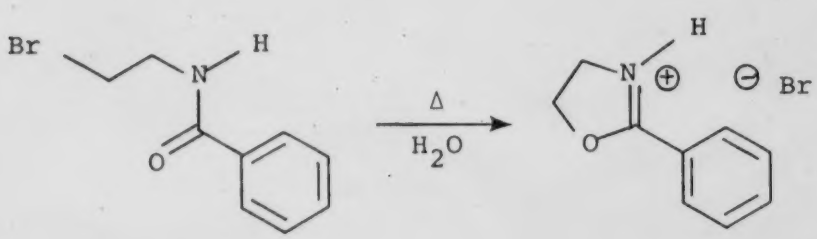
The basis for the alkylating activity of 3-(2-chloroethyl)cytidine 127 residues is unclear. One explanation for the necessary labilization of the carbon-chlorine

bond in such compounds is the formation of a short lived oxazolinium ion 147.



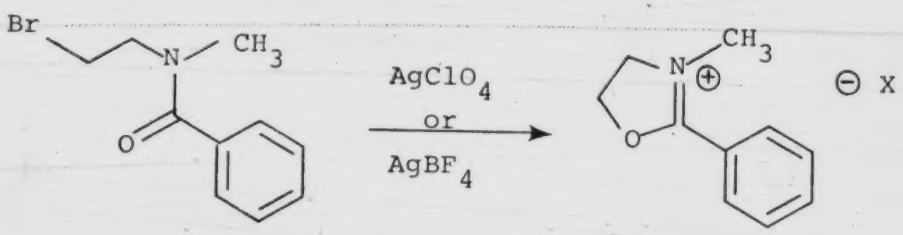
147 127 147

While there is no evidence that such an intermediate results from the reaction of chloroethyl nitrosoureas with DNA, there are literature precedents of the formation of simple oxazolinium ions by similar cyclization. Gabriel and Heymann reported[130] that β-bromoethyl benzamide 148 is unstable when heated in water and undergoes intramolecular cyclization to produce 2-phenyloxazoline hydrobromide 149.



148 149

β-chloroethyl benzamides are somewhat more stable but undergo similar cyclizations.[131] Kagiya *et al.*[132] reported the cyclization of N-(2-bromoethyl)-N-methyl-benzamide 150 using either silver perchlorate or silver tetrafluoroborate in nitrobenzene at room temperature.



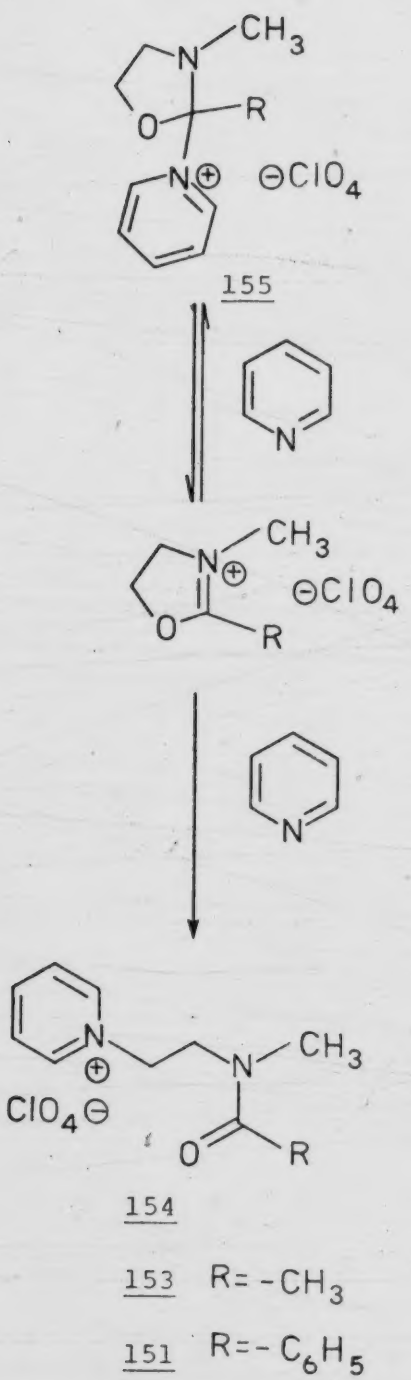
150

151 X = $ClO_4^{\ominus}$

152 X = $BF_4^{\ominus}$

They have also observed[132] that N-methyl-2-phenyl-2-oxazolinium perchlorate 151 or the similar N-methyl-2-methyl-2-oxazolinium perchlorate 153 are subject to ring opening. Nucleophilic ring opening of the oxazolinium ions by pyridine at room temperature proceeds *via* two competitive pathways producing the straight chain 154 and cyclic 155 pyridinium perchlorates in Figure 21. The former is a thermodynamically stable product and the latter a kinetically controlled product.

If the intermediate 147 was formed after chloroethylation of cytidine residues in DNA, the investigation by Kagiya[132] indicates that it should be more susceptible to nucleophilic attack than a simple alkylchloride.



154

153 R= -CH_3

151 R= -C_6H_5

Figure 21. Reaction of a pyridine nucleophile with model oxazolinium ion.

The reactions of 2-chloroethylnitrosoureas with DNA are envisaged to occur as shown in Figure 22. Initially the N^4-(2-chloroethyl)cytidine 141, 3-(2-chloroethyl)-cytidine 127 and 3-(2-hydroxyethyl)cytidine 125 derivatives are produced. The 2-chloroethyl derivatives can undergo intramolecular alkylation resulting in 3,N^4-ethano-cytidine 126 or intermolecular alkylation to form inter-strand or intrastrand cross-links.

Kohn[105] has suggested that a two carbon interstrand cross-link could only result between bases which normally base pair. Examination of space filling models shows that the 36° rotation between two adjacent G-C pairs in helical DNA allows the N^4 position of cytidine of one base pair to approach the O^6 position in guanosine such that a two carbon link between these positions imparts minimal distortion of the helix (Fig. 22). With the same base geometry it is also possible to create a two carbon cross-link between the N^4 position of a cytidine molecule and the 7-position of guanosine.

Subsequent work in this laboratory will involve the isolation of the cross-linked nucleosides produced by 2-chloroethylnitrosoureas. Shapiro[133,134] has recently reported general techniques which allow the isolation of modified nucleosides particularly those resulting from interstrand cross-linking.

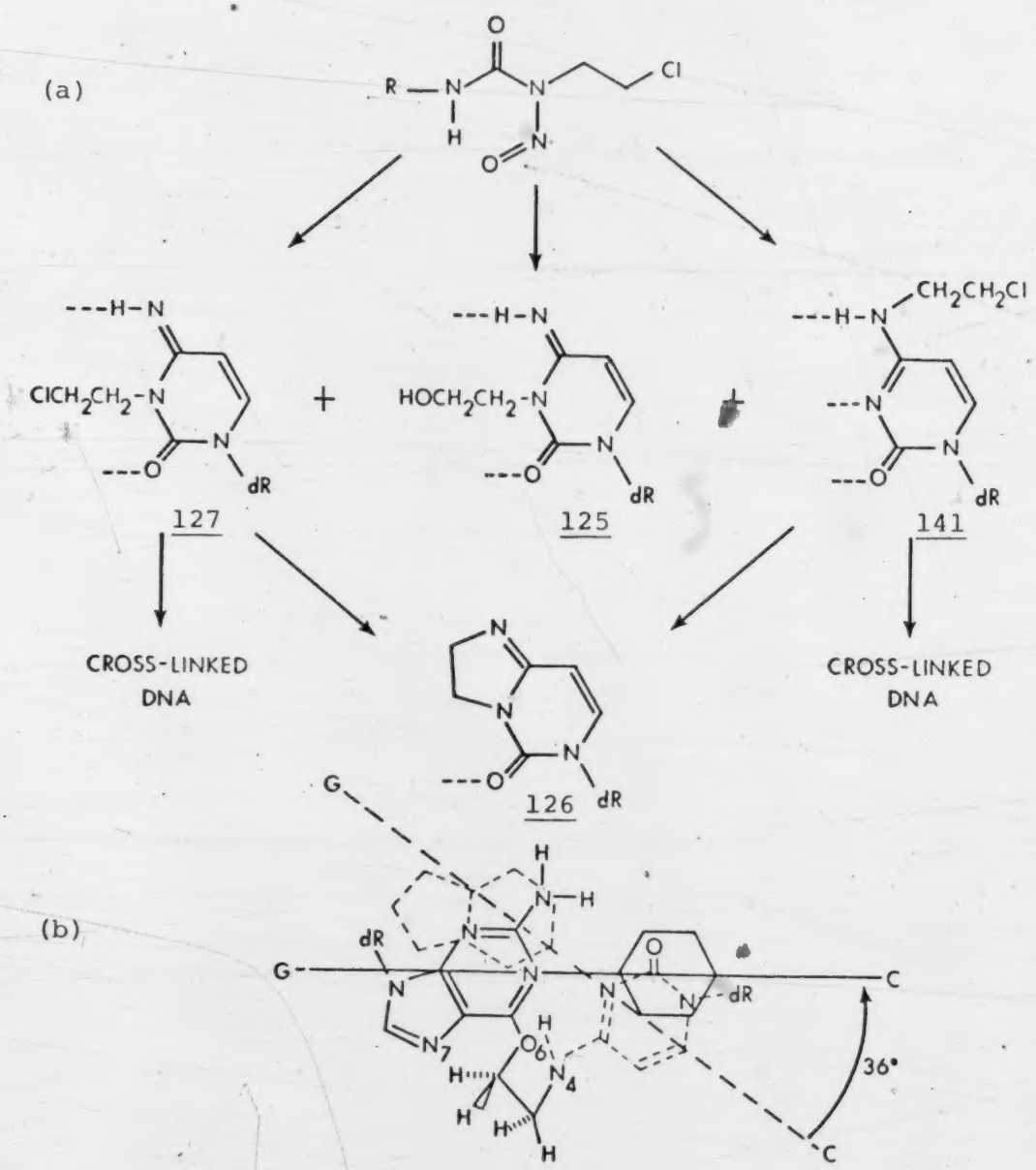


Figure 22. (a) Reactions of 2-chloroethylnitrosoureas with cytidine residues in DNA.

(b) Suggested sites for DNA cross-link.

Factors Affecting the Extent of DNA Cross-Linking.

The extent of interstrand cross-linked DNA produced by chloroethyl nitrosoureas is considerably lower than that observed for other bifunctional alkylating agents under comparable conditions[110,111,116,117] (compare CCNU 6 with mitomycin C 133, Table 12). Three processes could explain the observed low levels of DNA cross-linking.

(i) The extent of initial alkylation is low and thus the concentration of chloroethylated cytidine available for further alkylation is low.

(ii) Intramolecular alkylation or hydrolysis of chloroethylcytidine residues competes favorably with intermolecular akylation to form cross-links.

(iii) DNA degradation occurs concomitantly with alkylation and cross-linking and results in the extent of interstrand cross-linking appearing low.

(i) Studies Related to DNA Alkylation.

To measure the extent of chloroethylation of λ-DNA, ^{14}C-CCNU labelled in the 2-chloroethyl portion of the molecule was incubated with λ-DNA as described in the beginning of this chapter. As reported previously, at twice the concentration, the extent of alkylation by CCNU is much lower than that observed for mitomycin C (Table 12). The extent of alkylation increases in relation to the concentration of CCNU 6 as is expected. However, the percentage of cross-linked DNA is below 50% at binding

ratios which approach those of mitomycin C (where 84% cross-linked DNA results). These observations suggest that while a low extent of DNA alkylation may in part result in decreased interstrand cross-linking it is not the only contributing factor. Significant increases in drug concentration to produce extensively alkylated DNA did not result in greater than 50% cross-linked DNA.

(ii) Studies Related to Intramolecular Reactions of Chloroethyl Cytidine Derivatives.

The possibility that intramolecular alkylation or cyclization competes favorably with interstrand cross-linking for the intermediate chloroethylated cytidine residue was investigated by comparing the rates at which 3-(2-chloroethyl)-1-methylcytosine 156 and N^4-(2-chloroethyl)-1-methylcytosine 146 alkylate intramolecularly to form 3,N^4-ethan-1-methylcytosine 157 (Fig. 23). The cyclizations in aqueous solution buffered to pH 7.2 at 37°C were observed to follow first order kinetics (Fig. 24). The N^4-substituted derivative 146 has a half-life of 16 min. and complete conversion to the cyclic compound occurred within 150 min. The 3-substituted derivative 156 under the same conditions has a half-life of 53 min. The faster rate observed for N^4-(2-chloroethyl)-1-methylcytosine 146 may result from

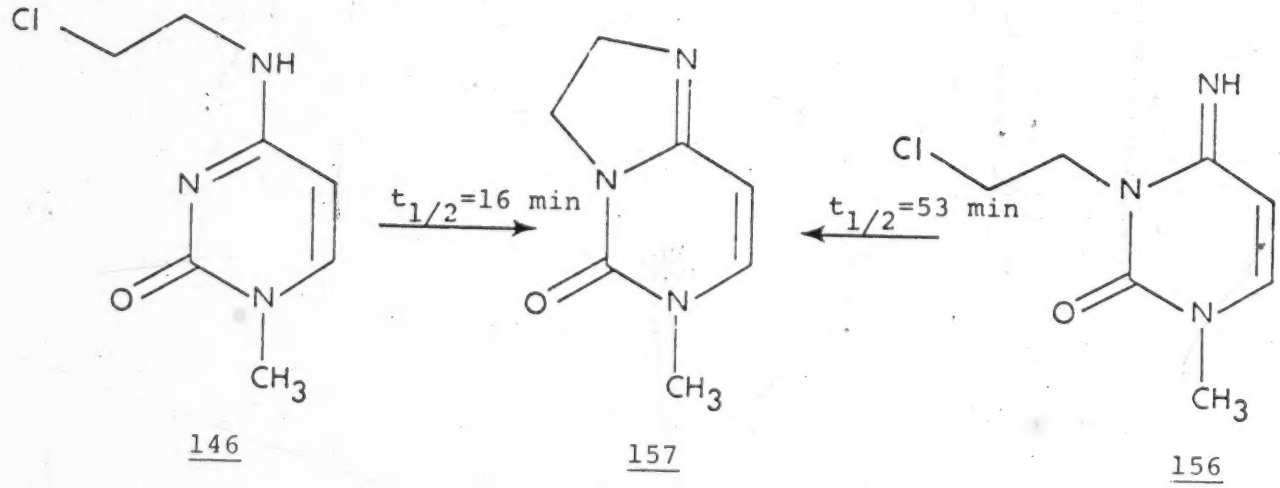


Figure 23. Conversion of 65 mM N^4-(2-chloroethyl)-1-methylcytosine or 3-(2-chloroethyl)-1-methylcytosine to 3,N^4-ethano-1-methylcytosine in 0.2 M phosphate buffer pH 7.2 at 37°C.

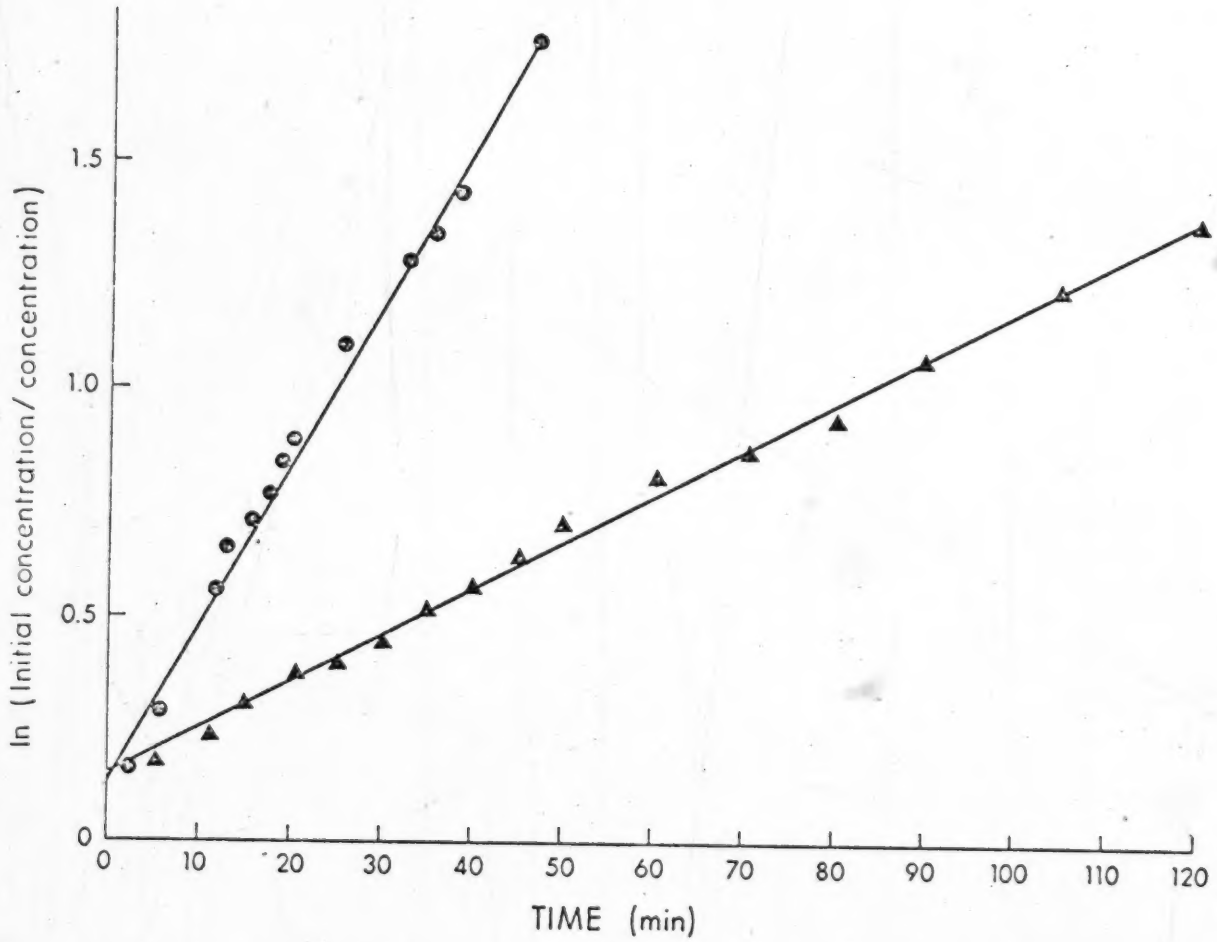


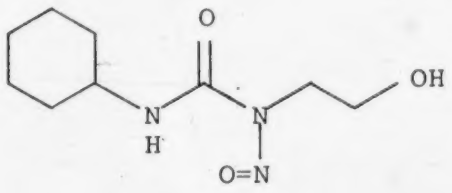
Figure 24. First order kinetic data for the formation of 3-N^4-ethano-1-methylcytosine from (●) N^4-(2-chloroethyl)-1-methylcytosine or (▲) 3-(2-chloroethyl)-1-methylcytosine.

the difference in nucleophilicities between the nitrogen and the exocyclic imino group. While similar reactions could occur in helical DNA after chloroethylation, slower rates for these reactions would be expected as the 3 or N^4 nitrogen may still be blocked by hydrogen bonding. Nevertheless, the results obtained for these model compounds suggest that after chloroethylation of cytosine bases in DNA, intramolecular alkylation may compete favorably with intermolecular alkylation and the formation of cross-links.

No hydroxyethylated cytosine was observed to be formed during either reaction. Although a 3-(2-hydroxyethyl)cytidine monophosphate 125 derivative has been isolated after treatment of poly C with BCNU 5,[81,102] the necessary hydrolysis of the chlorine atom appears to occur prior to alkylation. This observation is in accord with results published by Ludlum[135] concerning a 3-(2-fluoroethyl)cytidine derivative. A possible explanation for the formation of hydroxyethylated bases involves nucleophilic attack at the carbon bearing the nitrogen of the proposed oxadiazoline 106 (Fig. 11). While the amount of drug which results in hydroxyethylation from chloroethyl nitrosoureas cannot be estimated at this time, clearly such hydrolytic pathways will not result in the production of interstrand cross-links.

(iii) Studies Related to the Effects of Single Strand Scission on DNA Cross-linking.

The effects of single strand scission occurring concomitantly with cross-linking were then investigated. Previous studies have shown that chloroethyl nitrosoureas result in significant alkaline induced DNA degradation.[51-53] It will be shown in the following chapter that 2-hydroxyethyl nitrosoureas produce extensive single strand scission most probably through phosphate alkylation. Therefore, to determine the relative effects DNA single strand scission has on observed DNA interstrand cross-linking a compound mixture was prepared and assayed for cross-linking. Equivalent amounts of 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea (CHNU) 66 and BCNU 5 were incubated with λ-DNA at pH 7.2 and 37°C. The extent of



66

interstrand cross-linking for this mixture and that for BCNU 5 alone is observed in Figure 25. As can be observed in Figure 25, addition of the effective single strand scission agent CHNU 66 results in an apparent decrease in

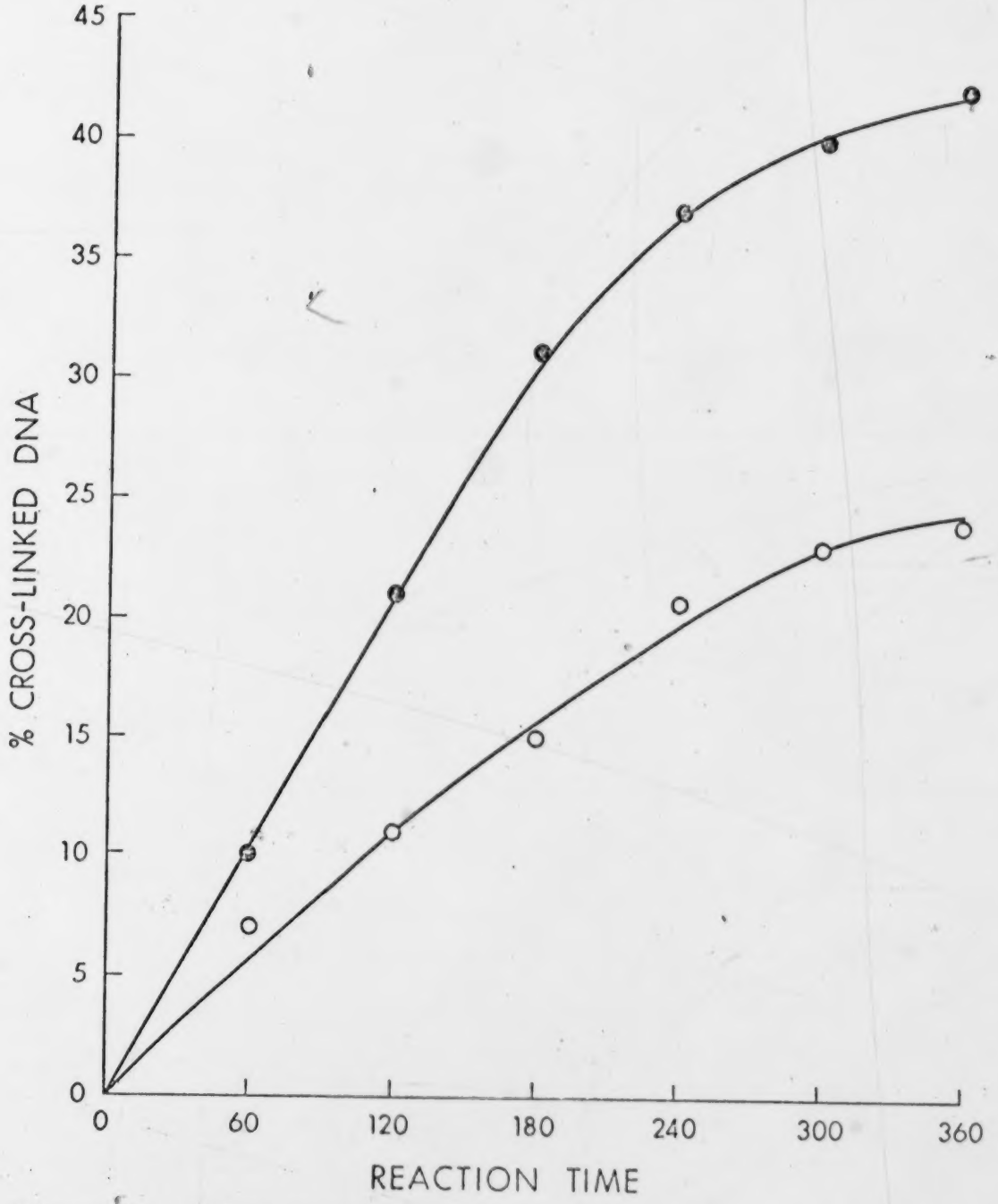


Figure 25. Reaction of λ-DNA 1.0 A_{260} in 0.05 M phosphate buffer pH 7.2 at 37°C with 5 mM: (●) BCNU 5 or (O) BCNU 5 plus 5 mM CHNU 66.

the extent of cross-linked DNA as compared to that observed for BCNU 5 alone. Cross-linking as measured spectrofluorometrically involves heat denaturation under alkaline conditions followed by renaturation of DNA which has been chemically cross-linked. Decreasing the molecular weight of the DNA by single strand scission results in smaller fragments of DNA renaturing after heating and an apparent decrease in cross-linked DNA.

Conclusions

DNA interstrand cross-linking has been observed for a number of nitrosoureas. It is most extensive with those containing a 2-chloroethyl moiety where it exhibits a pH dependence. Cross-linking is of a stepwise nature with the second alkylation completing the link occurring for up to 4-5 h. Fluoro-, bromo- and iodo- analogues, as well as chain branching or chain lengthening of chloroethyl nitrosoureas results in a low extent or loss of cross-linking activity which generally parallels anti-leukemic data.

Two model compounds, N^4-(2-chloroethyl)-1-methylcytosine 146 and 3-(2-chloroethyl)-1-methylcytosine 156 exhibit intermolecular DNA alkylation and implicate the analogous modified nucleosides in the cross-linking mechanism. The extent of interstrand cross-linking is lower than that observed for typical bifunctional alkylating agents.

Three processes are in part responsible for these low levels: (i) DNA alkylation by nitrosoureas is low, (ii) Intramolecular alkylation by probable intermediate chloroethyl bases competes favorably with intermolecular alkylation to produce cross-links, (iii) DNA single strand scission occurs concomitantly with cross-linking which reduces the apparent extent of cross-linking as measured spectrofluorometrically.

Experimental

Materials

^{14}C-CCNU was a gift from Dr. Gerald Goldenberg, Manitoba Institute of Cell Biology. 3-(2-adamantyl)-1-(2-chloroethyl)-1-nitrosourea 140 was supplied by Dr. Thomas Johnston, Kettering-Meyer Laboratory, Southern Research Institute. 1-(2-Chloroethyl)-3-phenyl-1-nitrosourea 135 and 1-(2-chloroethyl)-3-(2-chlorocyclohexyl)-1-nitrosourea (*cis* and *trans*) 138 and 139 were obtained from Dr. Harry B. Wood Jr., Division of Cancer Treatment, National Cancer Institute, Washington, D.C. PM2-CCC-DNA was a gift from Dr. A. Richard Morgan, Department of Biochemistry, University of Alberta. Ethidium bromide was purchased from Sigma and λ-DNA (m.w. 31 x 10^6) from Miles.

N^4-(2-Chloroethyl)-1-methylcytosine Hydrochloride 142.

This was prepared according to the method of Ueda and Fox.[125] It exhibited a double m.p. at 160° and 272-275°C (lit. m.p. 163-164 and 271-273°C). Pmr (D_2O) δ 4.8 (HOD): 3.22, 3.24 (s, 3H, CH_3); 3.7 (s, 4H, CH_2); 5.9, 6.2 (d, 1H, CH), 7.6, 7.8 (d, 1H, CH).

3,N^4-Ethano-1-methylcytosine Hydrochloride 158.

This was prepared according to the method of Ueda and Fox,[125] by heating a small amount of the above chloroethyl derivative on a heating block for a few

minutes at 170°C: m.p. 272-275°C (lit.[125] m.p. 271-273°C). Pmr (D_2O pH 7.2) δ 4.8 (HOD), 3.4 (s, 3H, CH_3); 4.2 (m, 4H, CH_2); 6.3 (d, 1H, CH); 8.0 (d, 1H, CH).

3-(2-Chloroethyl)-1-methylcytosine Hydrochloride 144.

3-(2-Hydroxyethyl)-1-methylcytosine prepared according to the method of Ukita *et al.*[136] was dissolved in ethanol saturated with HCl. After removal of the ethanol a 30 mg sample of the hydrochloride salt was added to 50 µl of thionyl chloride in 600 µl dry hexamethyl phosphoramide at 0°C. The solution was very slowly allowed to warm to room temperature and stirred overnight. 1 ml of ethanol was then added, the mixture was stirred an additional hour and then added to 7 ml of ether. The white solid was recrystallized twice from ethanol/ether without heating. Yield 15 mg (45%) m.p. 215°C and 269-272°C.

Anal. Calcd. for $C_7H_{10}N_3OCl \cdot HCl \cdot \frac{1}{4}H_2O$ [m.w. 187.0512 (free base)]: C, 36.77; H, 5.08; N, 18.38. Found (187.0519, mass spectrum): C, 36.63; H, 4.97; N, 17.99. Pmr (D_2O) δ (HOD 4.7); 3.5 (s, 3H, CH_3); 3.9 (t, 2H, CH_2); 4.5 (t, 2H, CH_2); 6.2 (d, 1H, CH); 7.8 (d, 1H, CH). Ir ν_{max} (EtOH) 3320 (N-H); 1630 (C=O); 1560 (C=N) cm^{-1}.

This compound could also be converted to 3,N^4 ethano-1-methylcytosine hydrochloride by heating a small amount on a heating block for a few minutes at 220°C. m.p. 269-272°C (lit.[125] m.p. 271-273°C).

Methods

Fluorescence Determination of Alkylation of PM2-CCC-DNA by Nitrosoureas.

A 20-µl aliquot was taken at intervals from the reaction mixture [50 mM potassium phosphate, pH 7.2, 1.2 A_{260} units of PM2-CCC-DNA (90% CCC), 5 mM nitrosourea in a total volume of 200 µl at 37°C] was added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium). The fluorescence after heating at 96°C/3 min followed by rapid cooling was compared with the initial value.

Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-CCC-DNA shows a decrease in fluorescence because of thermally induced depurination followed by alkaline strand scission of the apurinic site in the assay medium. The ratio of the decrease in fluorescence (after the heating and cooling cycle) to that of the control is a measure of the extent of alkylation. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence.

Binding of ^{14}C-CCNU to λ-DNA

Duplicate 100 µl mixtures containing the desired concentration of ^{14}C-CCNU were incubated at 37°C and pH 7.2 with 1.0 A_{260} λ-DNA (m.w. 31 x 10^6). After a 6 h

incubation a 10 µl aliquot was transferred to the assay solution and the extent of interstrand cross-linking was measured. A 1 µl aliquot was transferred to 10 ml of a toluene based liquid scintillation cocktail (Scinit-Verse, Fisher Scientific Co.) and counted on a Beckman LS 100c (serial # 1000930) scintillation counter using a ^{14}C-toluene standard (New England Nuclear, 4×10^5 dpm/ml) to determine initial CCNU concentration. The remaining reaction mixture was dialyzed at 4°C against three 1000 ml volumes of 20 mM potassium phosphate pH 7.0 containing 2 mM EDTA. The DNA nucleotide equivalent concentration of the dialysate was determined by U.V. absorption at 260 nm assuming an extinction coefficient of 7000. A 100 µl aliquot of the dialysate was then counted as described above to determine the concentration of DNA bound radioactivity. A 100 µl aliquot of the dialysis solution was used to determine background counts.

The ratio of initial CCNU concentration to DNA bound radioactivity (corrected for a 1.0 A_{260} DNA concentration) is used to determine the hydrolyzed drug/bound drug ratio. The ratio of DNA nucleotide equivalent concentration after dialysis to DNA bound radioactivity is used to determine the binding ratio.

Fluorescence Assay For Determining CLC Sequences in DNA Produced by Nitrosoureas.

All measurements were performed on a G. K. Turner and Associates Model 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100 x scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22°C. A 20-µl aliquot was taken at intervals from the reaction mixture (50 mM potassium phosphate, pH 7.2; 1.0 A_{260} units of λ-DNA; 5 mM nitrosourea; total volume, 200 µl) at 37°C and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium). The fluorescence after the heating and cooling cycle compared with control times 100 gives the percentage of CLC-DNA in a sample. For a standard set of conditions (i.e., type and concentration of DNA, pH, ionic strength, and the temperature), the accuracy of the CLC assay is determined by the precision of the fluorescence readings. Overall accuracy of the CLC assay is estimated at ±2%.

Stability of Interstrand Cross-Links.

A 300 µl sample containing 1.12 A_{260} units of λ-DNA, 40 mM potassium phosphate pH 8.6, and 10 mM BCNU was incubated at 50° for 3 hr and the extent of DNA cross-linking measured. The sample was dialyzed against 0.15 M NaCl and 0.015 M sodium citrate (known to reverse the cross-links of carzinophillin)[119,120] at 4° for 15 h. The sample was then incubated at 37°C for 48 h and the extent of cross-linking measured again.

Two-Step Nature of Cross-Linking of λ-DNA by Nitrosoureas.

A 400 µl sample containing 1.4 A_{260} of λ-DNA solution at pH 7.2 was prepared with BCNU or CCNU with the concentrations used above and incubated at 37°C for a period corresponding to two half-lives of decomposition of the nitrosourea. The reactions were quenched in ice and dialyzed against 50 mM potassium phosphate pH 7.2 at 4° for 15 h to remove unreacted nitrosourea. The dialysate was incubated at either 37° or 50° and assayed for cross-linking. The control consisted of nitrosourea and λ-DNA at 0°, dialysis as described, incubation at 37° or 50° and assaying for DNA cross-links.

Conversion of N^4-(2-Chloroethyl)-1-methylcytosine 146 To 3,N^4-Ethano-1-methylcytosine 157.

The pmr (D_2O pH 7.2) of the chloroethyl derivative shows a sharp resonance for the methylene protons at δ

3.6 (s, 4H). Under the same conditions the cyclized product exhibits a close A_2B_2 pattern centered at δ 4.25 (m, 4H).

A 1 ml D_2O reaction mixture containing 65 mM of the chloromethyl compound in deuterated 200 mM potassium phosphate pH 7.2 was incubated in the pmr probe of a Varian A 100 analytical spectrometer. The rate of intramolecular cyclization was obtained by monitoring the changes in the areas of the signals listed above.

Conversion of 3-(2-Chloroethyl)-1-methylcytosine 144 to 3,N^4-Ethano-1-methylcytosine 157.

The pmr (D_2O pH 7.2) of the chloroethyl derivative shows a resonance for one of the ring protons at δ 7.8 (d, 1H). Under the same conditions the cyclized product exhibits a similar doublet for one of the ring protons shifted slightly upfield. While the inner peaks of the two doublets ovelap the outer resonances are cleanly separated.

A 1 ml D_2O reaction mixture containing 65 mM of the chloroethyl compound in deuterated 200 mM potassium phosphate pH 7.2 was incubated in the pmr probe of a Varian A 100 analytical spectrometer. The rate of intramolecular cyclization was obtained by monitoring the changes in the areas of the signals listed above.

Dependence of Cross-Linking of Natural DNAs by Nitrosoureas on the (G + C) Content.

A 200 µl sample containing 50 mM potassium phosphate pH 8.6, 10 mM nitrosourea and 10% acetonitrile was incubated at 37°C with 1.6 A_{260} units of *Clostridium perfringens* DNA (30% G + C, m.w. 7.80×10^6); calf thymus DNA (40% G + C, m.w. 3.47×10^6); or *E. coli* DNA (50% G + C, m.w. 0.87×10^6). (The m.w.s were determined by sedimentation velocities). Assuming a Poisson's distribution of the cross-links and that one link is sufficient to permit spontaeous renaturation, the average number of cross-links per molecule M was determined from $M = \ln(1/Po)$ [where Po is the fraction of molecules not cross-linked[110]].

CHAPTER FOUR

NITROSOUREA INDUCED DNA SINGLE STRAND SCISSION

DNA damage in the form of alkali labile sites which produce single strand breaks has been reported for BCNU 5,[51,137] CCNU 6,[52,53] and MNU 2[138] as well as other methylating agents.[138] Kohn[51] and Gutin[52] have detected DNA degradation by BCNU 5 and CCNU 6, respectively using sedimentation rates through an alkaline sucrose gradient. In this assay, when nitrosourea treated DNA is exposed to alkali (0.1 N NaOH and 0.9 N NaCl) the strands begin to separate with the single strand breaks serving as points where unwinding can begin.[139] Complete unwinding between breaks results in the release of single stranded DNA fragments which sediment faster in an alkaline sucrose gradient.[140] Gutin[52] has attempted to quantitate DNA damage by its susceptibility to digestion by S_1 nuclease, a single strand specific nuclease from *Aspergillus oryzae*.[141,142] After treatment of the DNA with CCNU 6 and exposure to alkali it was subjected to the enzyme preparation. Enzyme resistant duplex regions of the DNA were detected using the fluorescence assay developed by Kissane and Robbins.[143] Hilton *et al.*[53] have used chromatography on hydroxylapatite to determine the extent of damage to DNA exposed to CCNU 6. After alkaline treatment, the separation of double stranded and single stranded DNA on hydroxylapatite was done according to the

method of Rydberg.[144] Recently Kohn[51,137] has used the rate of alkaline elution of cell lysates from a membrane filter after treatment with BCNU 5 to observe DNA damage. This technique relies on the relationship between DNA single strand length and the rate of elution of these strands from cell lysates at pH 12.1.[145] Small single stranded fragments elute very quickly while duplex DNA regions remain bound to the membrane filter.[145]

Although all of the techniques briefly discussed above allow the detection of alkali induced DNA degradation there are two major disadvantages, (i) A relative estimate of the number of single strand breaks is obtained but the chemical mechanisms cannot be easily investigated. (ii) Since the irreversible separation of single stranded DNA fragments is required in all of the above assays, compounds which cross-link DNA, in addition to causing DNA degradation, will inhibit the release of such single stranded fragments where they are involved in a chemical cross-link.

DNA single strand scission (SSS) can occur primarily through three processes:

(i) The generation of highly reactive radicals (superoxide or hydroxyl) in the vicinity of the DNA molecule results in the formation of DNA strand breaks.

(ii) Alkylation followed by depurination or depyrimidination results in labile apurinic sites. Three pathways are possible for the transformation of apurinic sites to

single strand breaks, (a) hydrolysis under alkaline conditions, (b) treatment with an appropriate amine, (c) enzymatic action.

(iii) Alkylation of the phosphate groups forms phosphate triesters which are susceptible to alkaline hydrolysis resulting in single strand breaks. This chapter considers these alternative pathways as they apply to the chemical mechanisms of the nitrosoureas.

The Detection of DNA Single Strand Scission Using the Ethidium Bromide Fluorescence Assay.

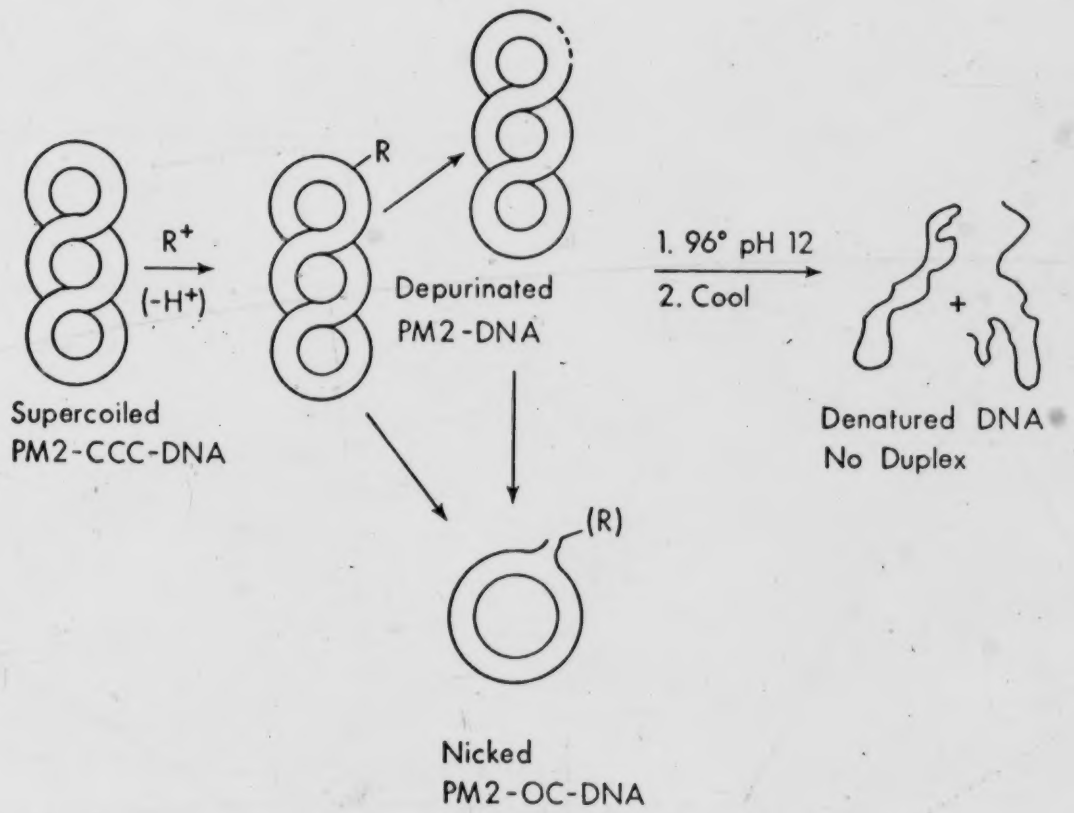
The fluorescence assay described in Chapter III has been extended to detect DNA single strand scission by making use of a covalently closed circular DNA (CCC-DNA). The amount of ethidium bromide 132 taken up by PM2-CCC-DNA is restricted due to topological constraints. If single strand scission of the CCC-DNA occurs in one or more places open circular DNA (OC-DNA) results in which the topological constraints are removed. OC-DNA takes up about 30% more ethidium than CCC-DNA with a corresponding increase in fluorescence.

PM2-CCC-DNA returns to register upon heating (96°C/3 min) and cooling (0°C), resulting in a fluorescence intensity which is the same as before the heating cooling cycle. In contrast PM2-OC-DNA, upon heating and cooling, denatures into one linear and one circular strand.

With no duplex regions remaining (pH 11.8) the fluorescence falls to zero. This is illustrated in Figure 26.

This assay is complicated when the scission agent also cross-links the DNA. With concomitant single strand scission and interstrand cross-linking the 30% fluorescence increase prior to the heating/cooling cycle is observed, however, due to the cross-linking, the two strands do not completely denature after heating and cooling. The fluorescence no longer falls to zero but instead reflects the extent of DNA interstrand cross-linking. While the 30% increase in fluorescence can only be accounted for by cleavage of CCC-DNA to OC-DNA, a 30% range was not sufficient to allow a detailed study of the molecular mechanisms involved.

The increase in fluorescence due to the conversion of PM2-CCC-DNA to PM2-OC-DNA can be further enhanced by initially treating the PM2-CCC-DNA with the calf thymus topoisomerase.[146] Native PM2-CCC-DNA contains negative supercoils.[147] The topoisomerase by acting sequentially as both an endonuclease and a ligase removes the supercoils to relax the DNA.[146] During this process the number of intercalation sites for ethidium (which itself unwinds the supercoiled PM2-CCC-DNA) is decreased. The relaxation process can be monitored by a ~33% decrease in fluorescence. The conversion of relaxed PM2-CCC-DNA to PM2-OC-DNA now results in ~100% increase in fluorescence.



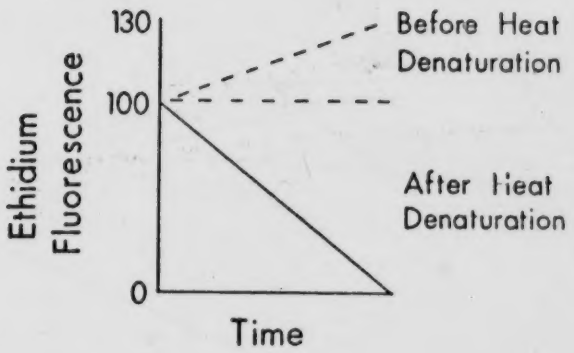


Figure 26. Ethidium bromide assay for the detection of single strand scission of supercoiled PM2-CCC-DNA.

DNA single strand scission can then be monitored in the presence of concomitant DNA interstrand cross-linking which has no effect on the fluorescence prior to the heating/cooling cycle. The use of the ethidium bromide fluorescence assay in conjunction with calf thymus topoisomerase is illustrated in Figure 27.

Detection of Type I Single Strand Scission (Type I SSS) and Type II Single Strand Scission (Type II SSS).

Extensive formation of alkali labile sites which result in single strand breaks in DNA is observed after treatment of relaxed PM2-CCC-DNA with nitrosoureas. The lesions produced in the DNA have much greater stability when the pH 7.2 ethidium assay is employed, while under alkaline assay conditions, pH 11.8, they are readily cleaved. The different rates of production of DNA single strand breaks observed in the alkaline assay solution, after treatment with nitrosoureas, suggest that at least two mechanisms are operative. There is an extremely fast reaction resulting in single strand breaks which occurs immediately after addition of an aliquot of nitrosourea treated DNA to a 20 mM pH 11.8 phosphate buffer at 22°C (Fig. 28). The extent of this type I single strand scission (SSS) increases with the time of reaction between DNA and drug (Fig. 29). Significant differences in the extents of type I SSS are observed

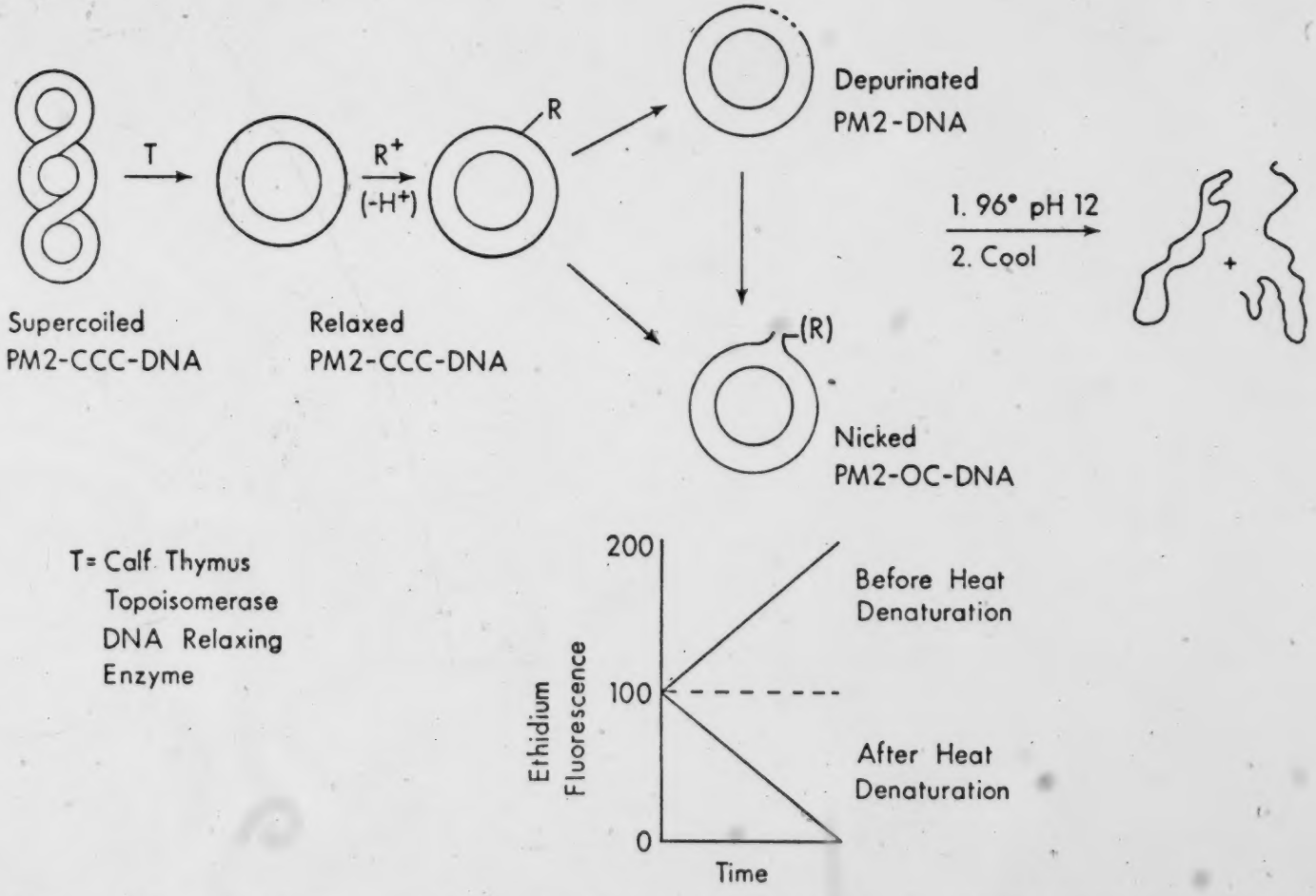


Figure 27. Ethidium bromide assay for the detection of single strand scission of PM2-CCC-DNA relaxed with calf thymus topoisomerase.

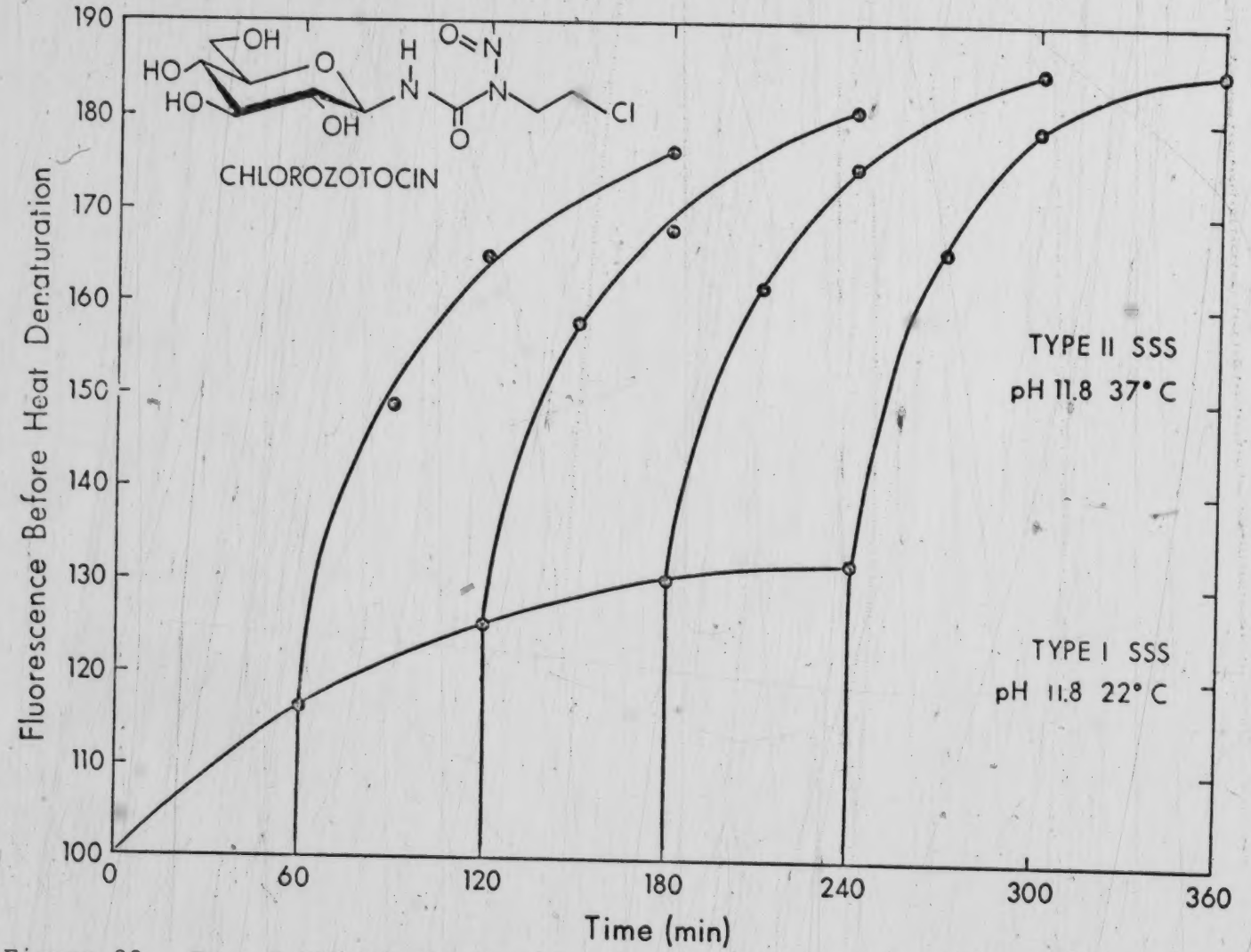


Figure 28. Type I DNA single strand scission (SSS) and Type II SSS. Reaction mixture contained relaxed PM2-CCC-DNA 1.0 A_{260} in 0.05 M cacodylate buffer pH 7.0 at 37°C and 5 mM chlorozotocin 86.

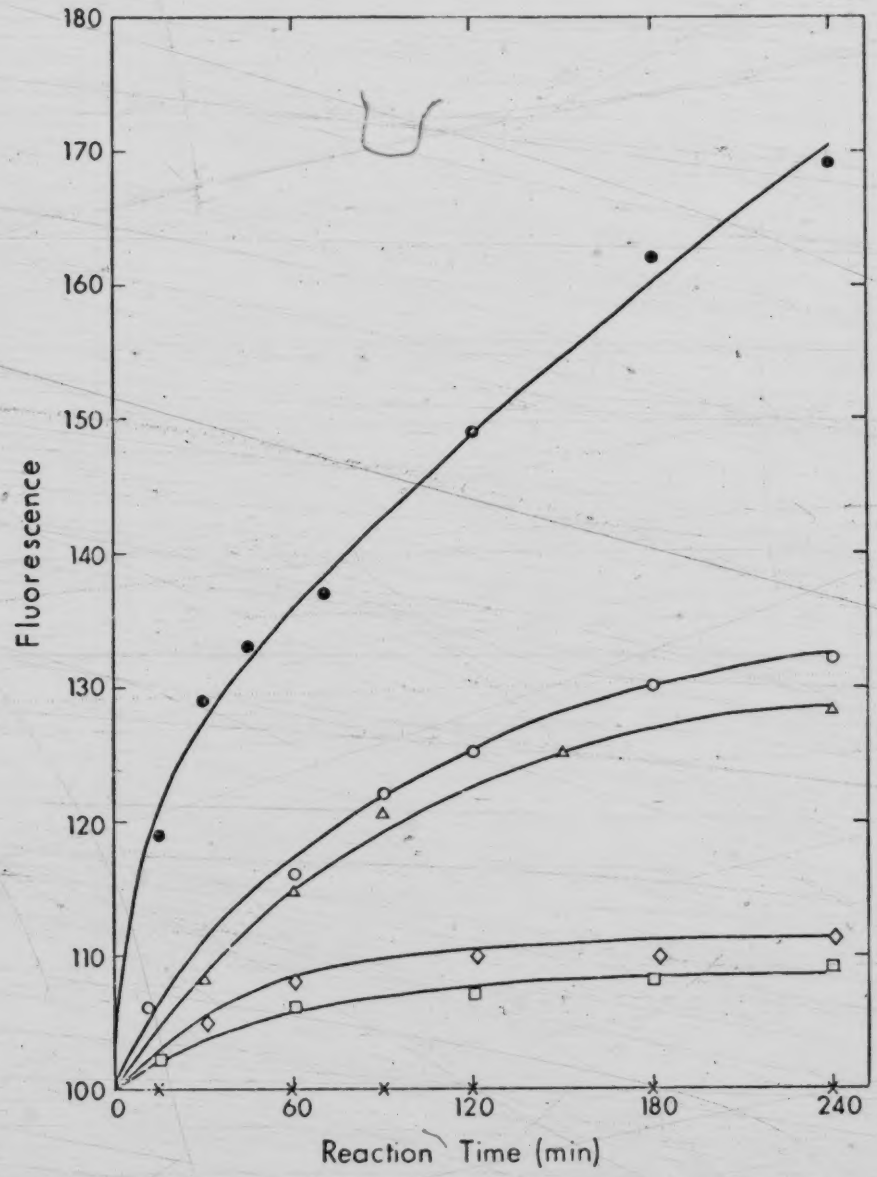
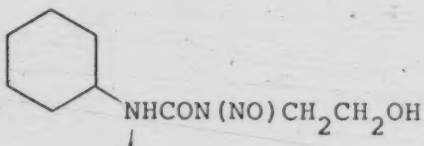


Figure 29. Type I SSS. Reaction of PM2-CCC-DNA 1.0 A_{260} in 0.05 M cadodylate buffer pH 7.0 at 37°C with 5 mM drug. Fluorescence values were obtained within 30 sec of addition of a 20 µl aliquot to the pH 11.8 assay solution at 22°. Type II SSS was not observed at 22°. (●) CHNU 66; (○) chlorozotocin 86; (△) BCNU 5; (□) ENU 10; (◇) BFNU 26; (X) Dimethyl sulfate 159 or 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea 67 or control (containing relaxed PM2-CCC-DNA with no drug).

for 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea (CHNU) 66, BCNU 5, BFNU 26 and ENU 10.



66

$ClCH_2CH_2NHCON(NO)CH_2CH_2Cl$

5

$FCH_2CH_2NHCON(NO)CH_2CH_2F$

26

$H_2NCON(NO)CH_2CH_3$

10

An additional slower production of single strand breaks is observed when the nitrosourea treated DNA is allowed to incubate at 37°C and pH 11.8 (Fig. 28). This type II process can be observed to occur for 90-120

minutes after addition to the pH 11.8 buffer (Fig. 30). Controls run with untreated DNA indicate its stability to the high pH conditions for 120 minutes. Dilution of the reaction mixture by the assay solution was observed to quench further reaction between DNA and unreacted nitrosourea. The presence of ethidium bromide during the detection of type II SSS did not significantly affect the observed rates.

Neither of the two processes was affected by the presence of enzymatic radical trapping agents such as superoxide dismutase and catalase. Chemical radical traps such as isopropyl alcohol and sodium benzoate also had no effect on the scission phenomenon. No strand scission is observed during the first four hours of reaction when a pH 7.2 assay solution is used. These observations rule out a radical process, similar to that which has been observed for drugs such as bleomycin and the anthracyclines in the presence of reducing agents[148,149] to account for the observed DNA degradation in the case of the nitrosoureas.

Studies Related to Type II SSS.

Nitrosoureas have been observed to alkylate the bases of nucleic acids.[15] The cytosine and guanine residues are reported to be most extensively alkylated.[15] A number of modified nucleosides have been isolated after treatment

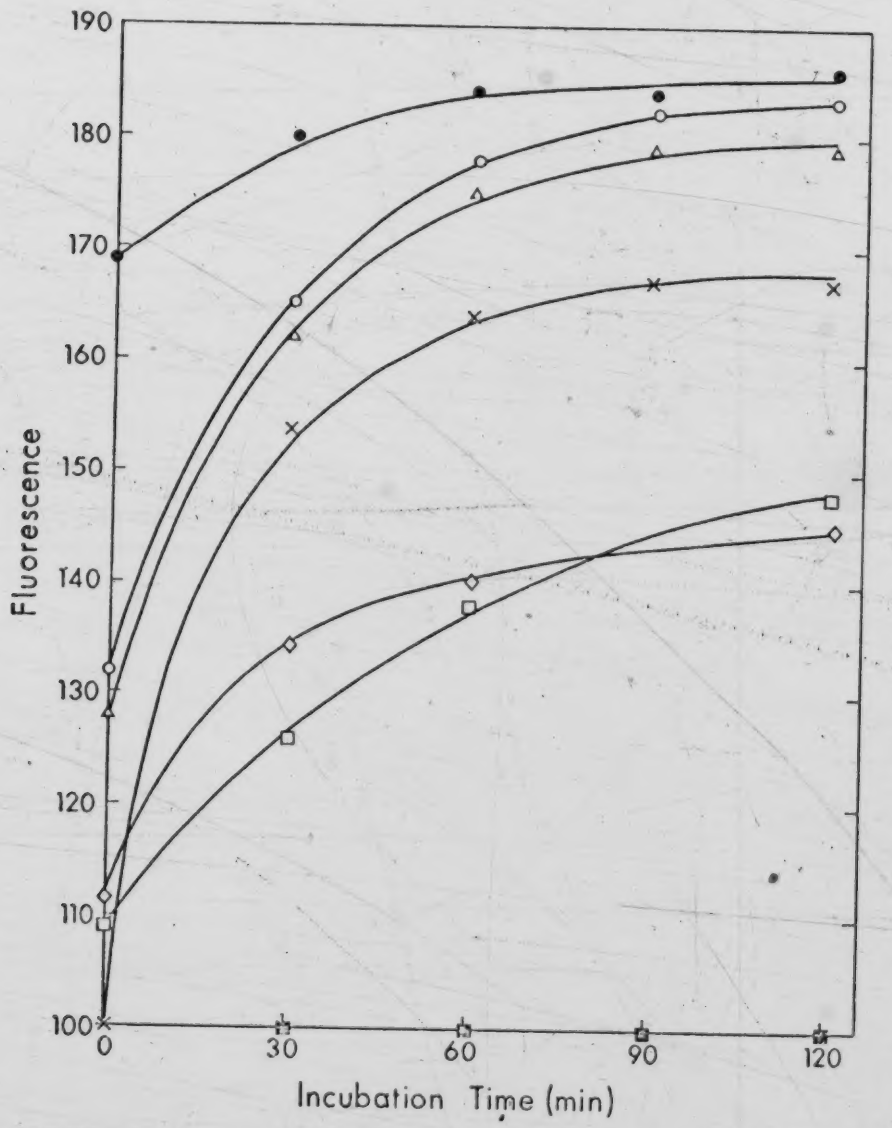


Figure 30. Type II SSS observed after 240 min of reaction of relaxed PM2-CCC-DNA 1.0 A_{260} in 0.05 M cacodylate buffer pH 7.0 at 37°C with 5 mM: (●) CHNU 66; (O) chlorozotocin 86; (△) BCNU 5; (◇) BPNU 26; (□) ENU 10; (X) dimethyl sulfate or (■) control: relaxed PM2-CCC-DNA with no drug. Sample incubation occurred in 0.02 M phosphate buffer pH 11.8 at 37°C. Assay solutions cooled to 22°C before fluorescence reading.

of synthetic polynucleotides with BCNU 5 which indicate that a β-chloroethyl or β-hydroxyethyl alkylating moiety has been transferred to the base.[81,102] The facile loss of alkylated bases from the modified DNA polymer to produce labile apurinic sites is well documented[92,150] and accounts for the depurination or depyrimidination observed in the present study.

To determine the contribution of the depurination-depyrimidination strand scission pathway to the overall degradation of nitrosourea treated DNA, the effects of dimethyl sulfate 159 were first studied. Dimethyl sulfate 159 is known to alkylate DNA extensively with the principle sites of attack occurring at the N-7 position of guanine and the N-3 position of adenine[92,150,151] with no oxygen alkylation of the bases or phosphates.[152] It has also been observed that N-3 alkylated adenine and N-7 alkylated guanine residues are readily lost to yield apurinic sties,[153] which, while stable under neutral conditions are subject to alkaline hydrolysis and the formation of DNA single strand breaks.[154] While dimethyl sulfate 159 treated relaxed PM2-CCC-DNA showed none of the type I SSS, extensive type II SSS was observed upon its incubation for 90 min at 37°C and pH 11.8 (Fig. 31).

Confirmation that the type II SSS observed for dimethyl sulfate 159 was due to production of apurinic sites and subsequent hydrolysis was obtained using an

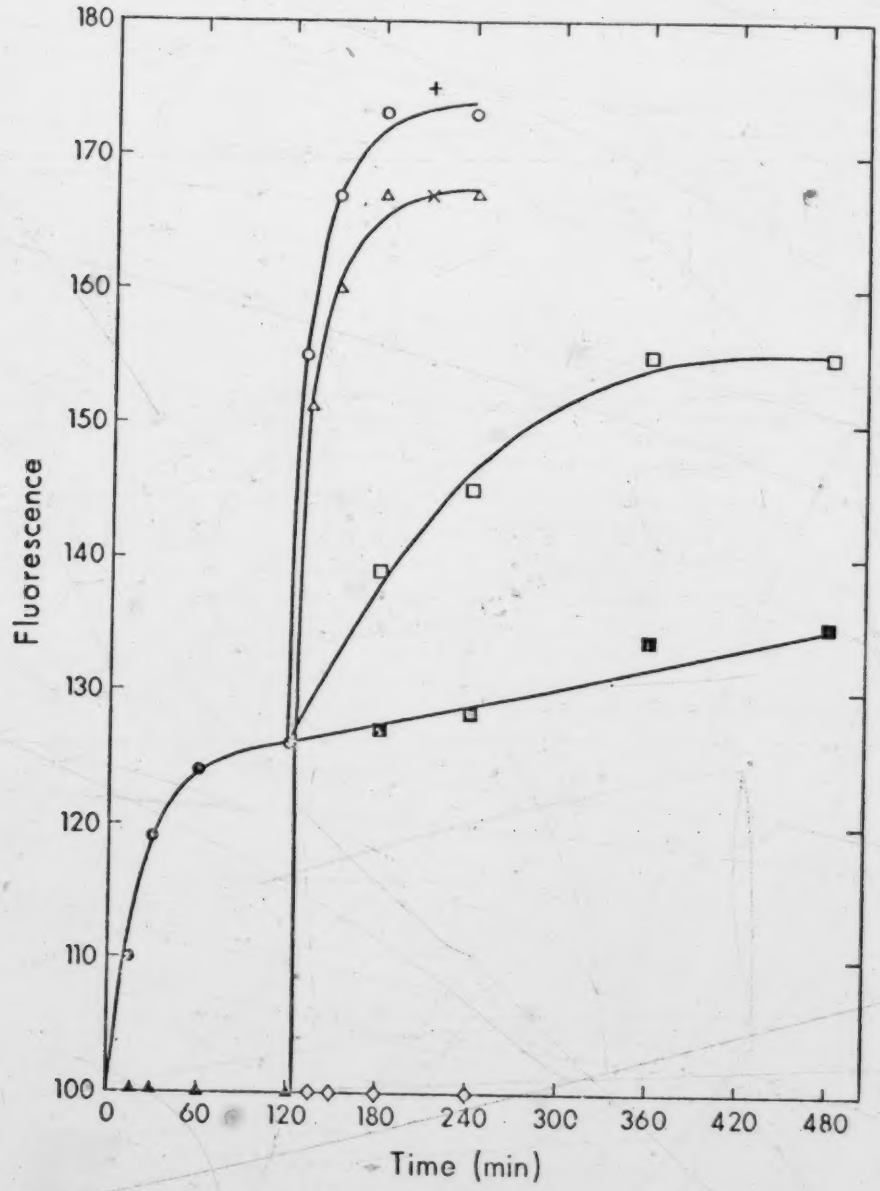


Figure 31. Reaction of PM2-CCC-DNA 1.0 A_{260} in 0.05 M cacodylate buffer pH 7.0 at 37°C with 5 mM drug. Measurement of type I SSS for (●) CNU 3, followed after 120 minutes of reaction with; (O) endonuclease VI; (+) 90 minutes incubation at 37° pH 11.8; (□) 5 mM aniline, (■) 5 mM cyclohexylamine. Measurement of type I SSS for (▲) dimethyl sulfate 159 followed after 120 minutes of reaction with (Δ) endonuclease VI; (X) 90 minutes incubation at 37° pH 11.8; (◇) control (relaxed PM2-CCC-DNA with endonuclease VI).

apurinic site specific endonuclease. Endonuclease VI first isolated by Berly and Rassart[155] recognizes apurinic sites and hydrolyzes the DNA sugar backbone at such points. Treatment of relaxed PM2-CCC-DNA with dimethyl sulfate 159 for a period of two hours was followed by treatment with the endonuclease VI. An immediate and extensive production of single strand breaks was observed indicating the existence of apurinic sites produced by dimethyl sulfate 159 (Fig. 31). Treatment of native supercoiled or relaxed PM2-CCC-DNA with the endonuclease was run as a control to show that the enzyme had no effect on the native DNA. Additionally it was observed that the rate of the type II SSS process is comparable with the rate of hydrolysis of apurinic DNA (generated under low pH conditions[156,157]) at 37°C and pH 11.8 (Fig. 32).

A similar study was then undertaken for 2-chloroethyl-nitrosourea (CNU 3). CNU 3 was chosen for two reasons, (i) the half life for CNU 3 at 37°C and pH 7.2 is approximately 9 minutes (see Chapter II) which compares favorably with the 10 minute half life observed for dimethyl sulfate 159 under similar conditions.[158] (ii) In addition to an alkylating moiety CNU produces isocyanic acid which is converted to sodium cyanate in cacodylate buffer (scheme 4). The N-3 substituted nitrosoureas decompose to form isocyanates which then hydrolyze to amines. It

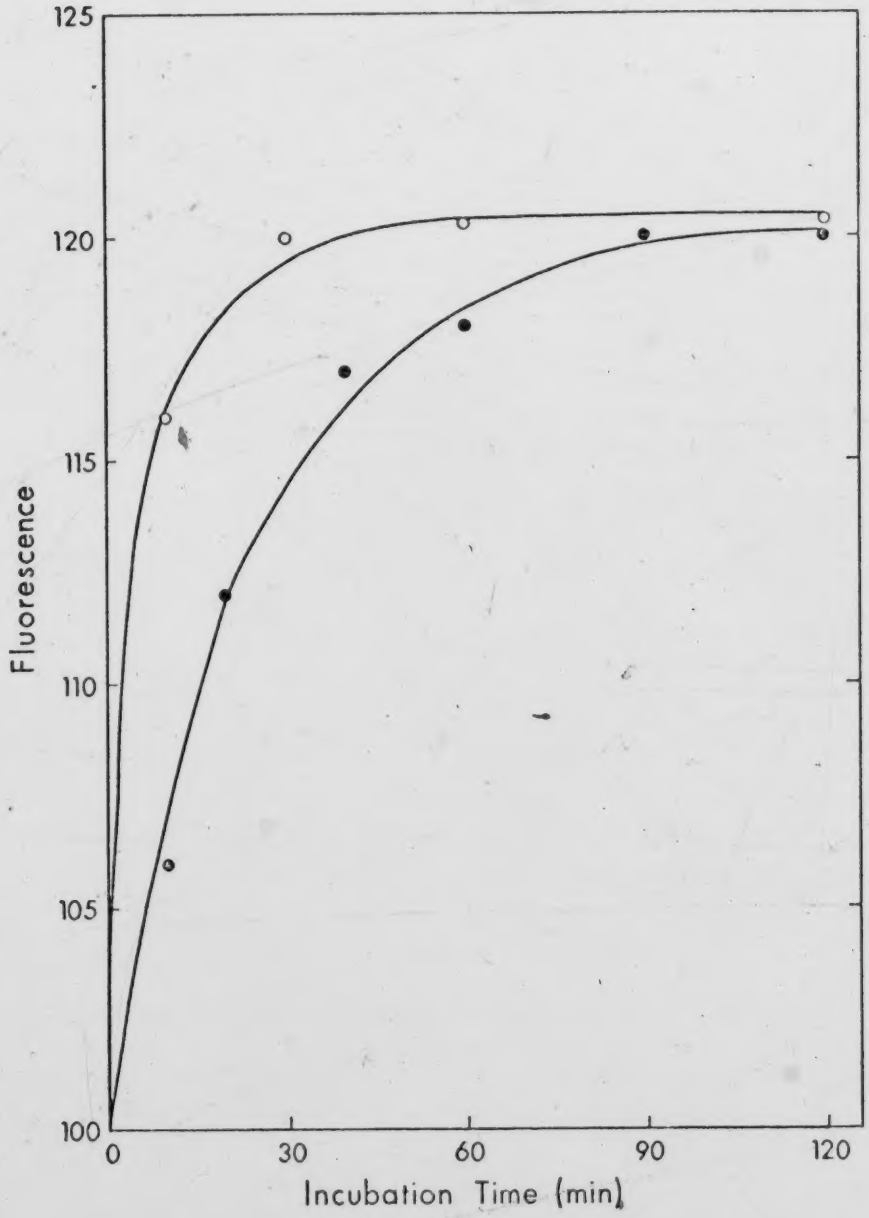
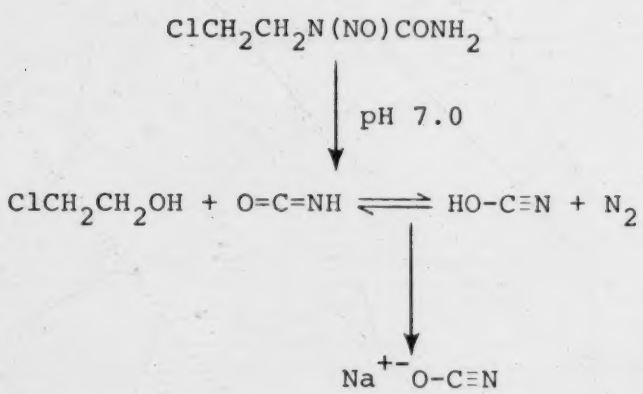


Figure 32. Alkaline catalyzed strand scission. 20 µl of 1.0 A_{260} apurinic PM2-CCC-DNA (generated by: (●) low pH; (○) treatment with dimethyl sulfate) incubated in 2 ml of the pH 11.8 assay solution at 37°C. Solutions cooled to 22°C before fluorescence readings.

Scheme 4



has been observed that amines react with apurinic sites,[159,160] a process which will be discussed shortly. The use of CNU 3 removed the possibility of this competing pathway during the initial study.

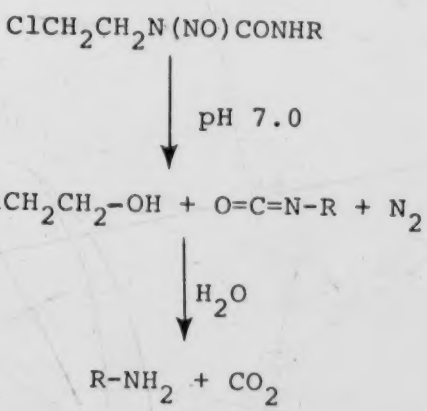
After reaction of CNU 3 with relaxed PM2-CCC-DNA for a period of 120 minutes significant type I SSS was observed (Fig. 31). Incubation of the reaction mixture for 90 minutes at pH 11.8 and 37°C indicated a considerable amount of type II SSS had also taken place. Confirmation that the type II SSS observed for CNU 3 was due to the formation of apurinic sites was again obtained using endonuclease VI (Fig. 31).

It is clear that apurinic sites lead to strand breaks under enzymatic treatment or alkaline hydrolysis. The

third possibility involves the reaction of apurinic sites with amines. It has been reported that the reaction of apurinic acid with an aromatic amine in the presence of aqueous formic acid results in DNA chain scission.[159,160] We have observed that at pH 7.2 there is significant reaction between aromatic amines and apurinic acid leading to strand scission while aliphatic amines show little or no reaction (Fig. 33). The difference presumably reflects the relative stabilities of the Schiff's bases formed.[161] This was substantiated by observing the differences in ability to cause strand scission by an aromatic amine containing either an electron withdrawing substituent or an electron donating substitute (Fig. 33).

An investigation was then initiated to determine if hydrolysis of apurinic sites by amines was resulting from N-3 substituted nitrosoureas (scheme 5) was a

Scheme 5



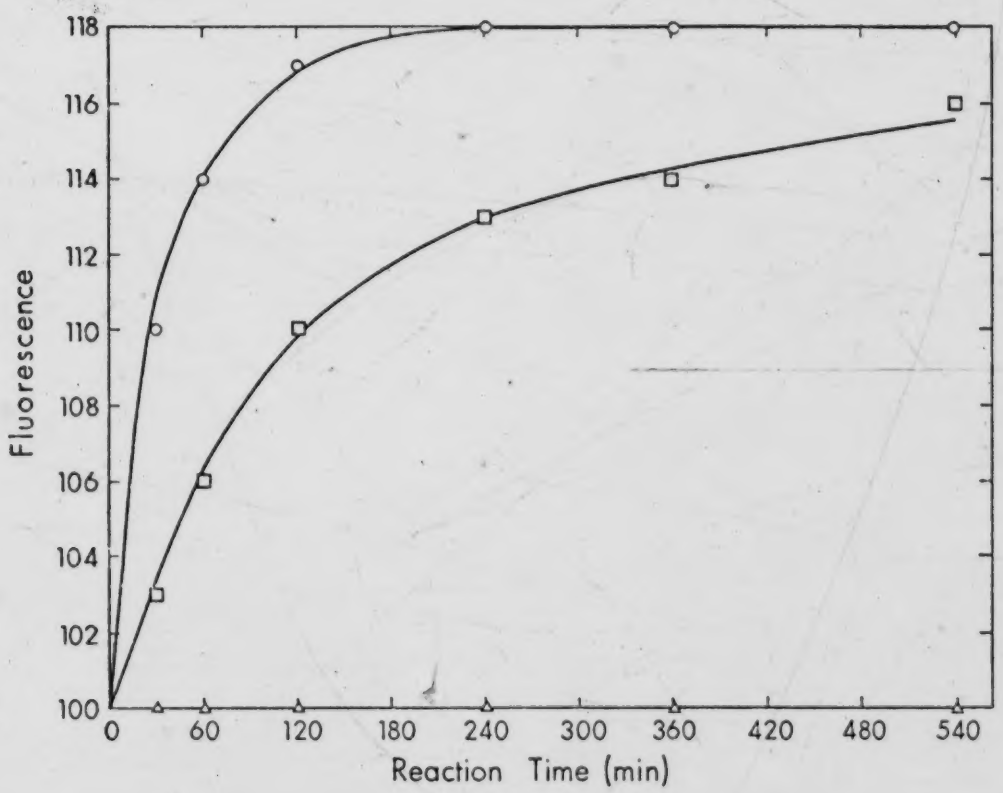
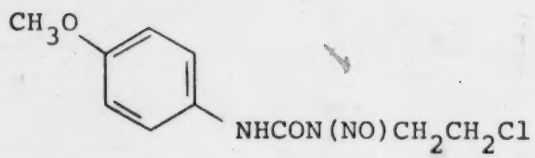


Figure 33. Reaction of apurinic PM2-CCC-DNA incubated at 37°. pH 7.2 with: 5 mM (O) p-methoxyaniline; (□) aniline; (△) p-nitroaniline, or cyclohexylamine or control. Fluorescence values obtained within 30 sec of addition of 20 µl aliquot to pH 11.8 assay solution.

contributing pathway in the degradation of nitrosourea treated DNA. CNU 3 was reacted with relaxed PM2-CCC-DNA for a period of 120 minutes. Type I SSS was observed when no amine was present in the reaction mixture. After two hours of reaction, an equivalent concentration of either cyclohexylamine or aniline was added to the nitrosourea DNA mixture. While aniline cause a significant further increase in single strand breaks during the following five hours, cyclohexylamine showed only a small additional effect upon DNA degradation (Fig. 31).

Reactions of amines with apurinic sites is unlikely to be a contributing pathway for nitrosoureas which produce aliphatic amines from isocyanate hydrolysis. Specifically, the type I cleavage phenomenon does not reflect amine-apurinic site reaction in the case of BCNU 5, CCNU 6, CHNU 66 or similar derivatives. However, it may be significant in the case of aryl substituted nitrosoureas. The relative extent of this contribution to the type I process was measured using three aryl derivatives: 1-(2-chloroethyl)-3-(p-methoxyphenyl)-1-nitrosourea 68, 1-(2-chloroethyl)-3-phenyl-1-nitrosourea 135 and 1-(2-chloroethyl)-3-(p-nitrophenyl)-1-nitrosourea 69. An ethidium bromide fluorescence assay for the ability



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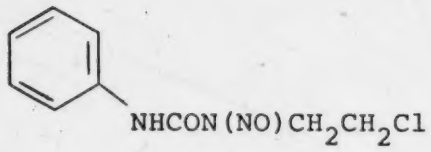

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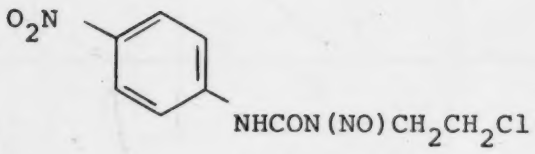

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of the three derivatives to initiate type I SSS is shown in Figure 34. The analogue producing the electron rich aryl amine and thus the more stable Schiff's base shows extensive type I SSS. The derivative with the electron withdrawing substituent on the aryl moiety shows little DNA strand scission, while the unsubstituted aryl derivative is intermediate in reactivity.

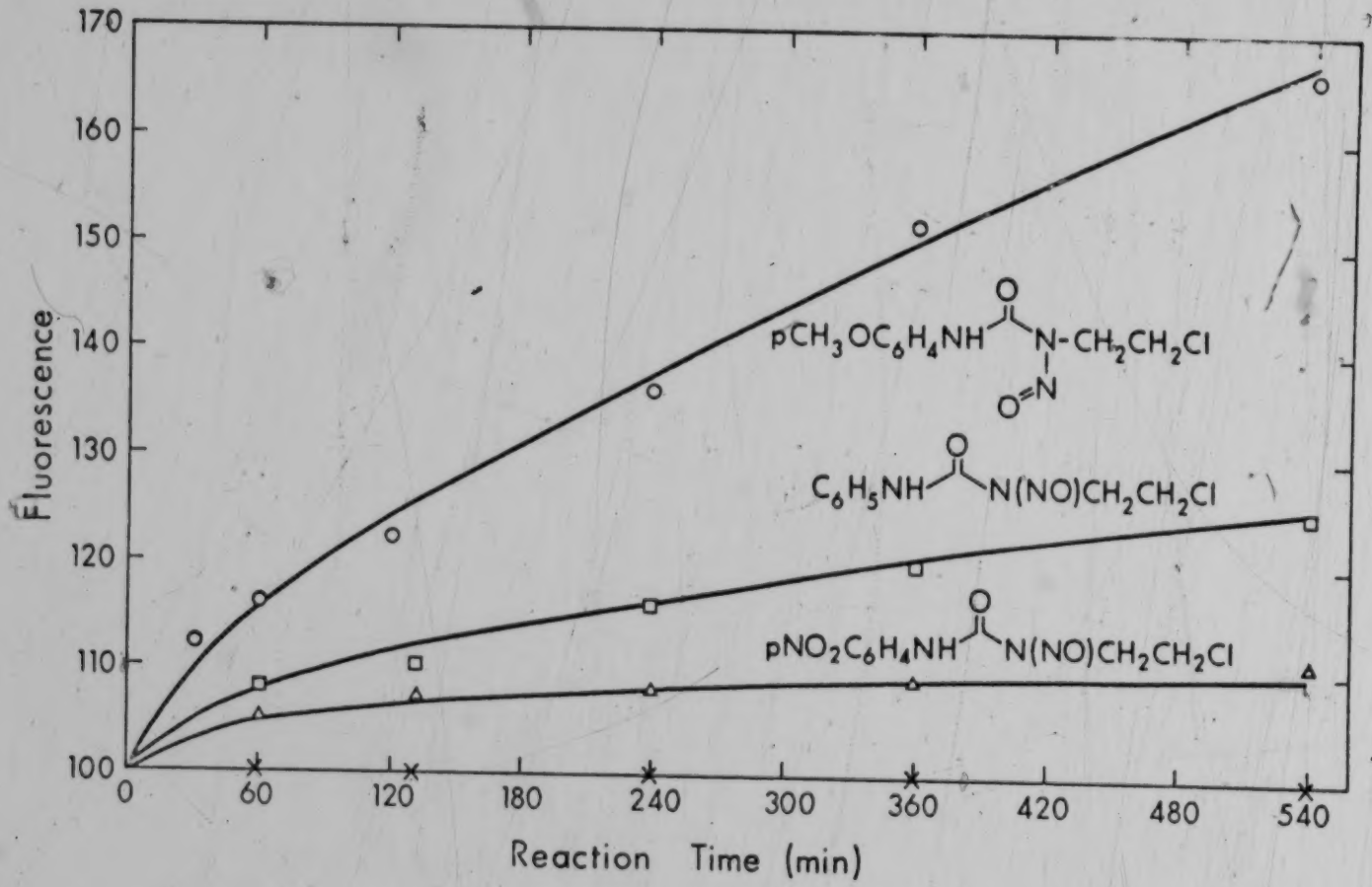


Figure 34. Type I SSS. Reaction of relaxed PM2-CCC-DNA 1.0 A_{260} in 0.05 M cacodylate buffer pH 7.0 at 37°C with 5 mM: (O) 1-(2-chloroethyl)-3-(p-methoxyphenyl)-1-nitrosourea 68; (□) 1-(2-chloroethyl)-3-phenyl-1-nitrosourea 135; (△) 1-(2-chloroethyl)-3-(p-nitrophenyl)-1-nitrosourea 69; (X) control, p-methoxy aniline.

Strand scission resulting from depurination of an alkylated base followed by reaction with an amine is envisaged to occur as shown in Figure 35. Loss of the alkylated base produces the apurinic site 160. Amines react with the open chain form of the deoxyribose 161 to produce the Schiff's base 162. Tautomerization of the Schiff's base to the enamine form 163 allows elimination of the phosphate from the 3' position of the sugar and results in DNA sugar backbone cleavage.

Studies Related to Type I SSS.

It has been previously shown by Singer *et al.* that when DNA is treated with ethyl nitrosourea 10, 65% of the alkylation events occur on the phosphate residues.[95,98] The resulting phosphotriesters, while known to be stable under neutral conditions,[33] have been observed to undergo base catalyzed hydrolysis.[162,163] Based on these observations, we initiated a study to determine if the nitrosoureas of interest in this work alkylated phosphate residues of nucleic acids. The obvious differences in the type I SSS observed for an ethylating agent, a chloroethylating agent and a hydroxyethylating agent (Fig. 29) dictated the three types of compounds that should be studied.

Verly and Bannon[33] have observed that ethyl and methyl phosphotriesters of DNA are stable under neutral

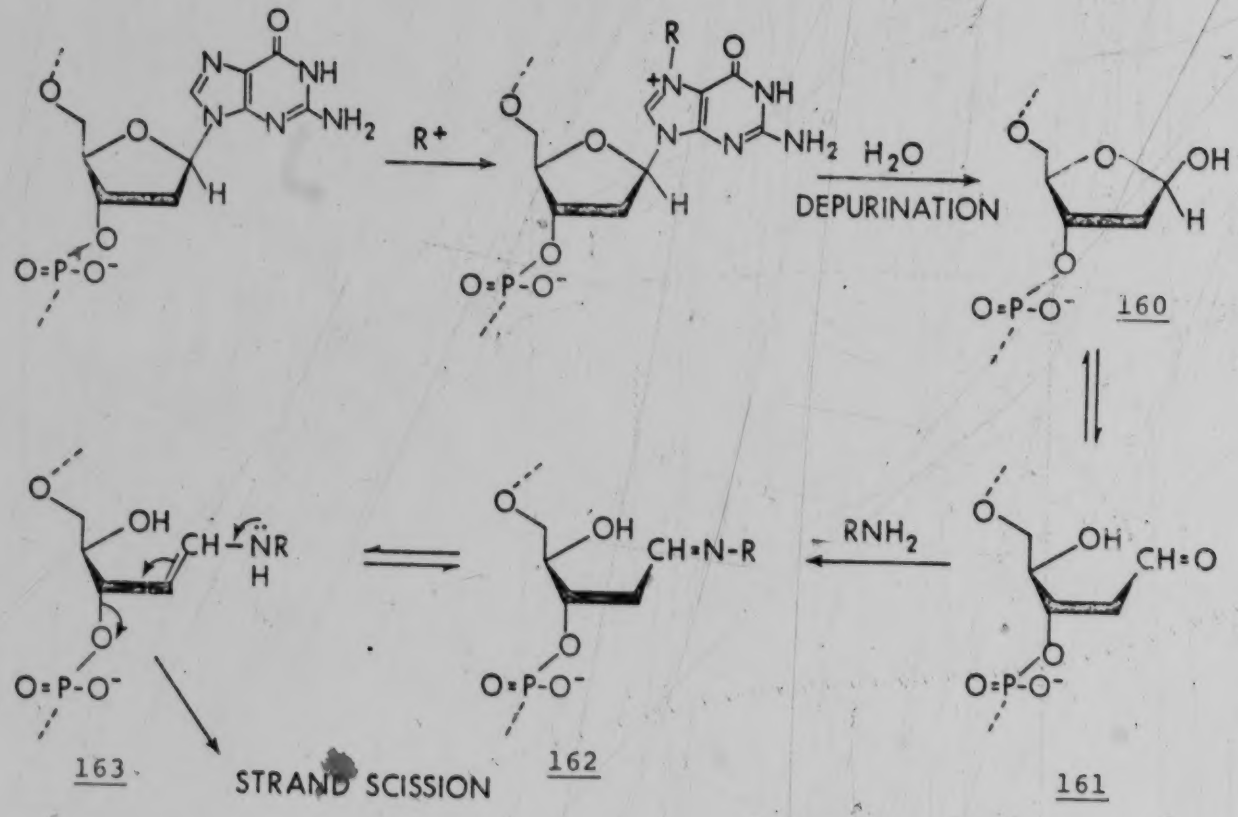


Figure 35. Suggested mechanism for amine catalyzed transformation of an apurinic site resulting from DNA alkylation, to a single strand internucleotide break.

conditions, while Shooter has reported that ethyl phosphotriesters hydrolyze only very slowly in 0.1 N NaOH.[163] RNA internucleotide linkages are much less stable and the glycosidic linkages much more stable than those in DNA. Phosphotriesters of ribonucleotides are unstable over the entire pH range presumably due to participation in the hydrolysis step by the 2'-hydroxyl group on the sugar moiety (Fig. 36).[164] This property has permitted observation of RNA degradation by alkylating agents to be used as a diagnostic test for phosphotriester formation.[165,166] Alkylation of the base residues of RNA produces a much more stable system than in DNA, and therefore, depurination of alkylated bases followed by hydrolytic cleavage of the apurinic site is less likely to contribute to RNA degradation. Therefore, it appeared that the best analytical method for the measurement of phosphate alkylation in nucleic acids involved monitoring molecular weight changes in RNA after treatment with the alkylating agent.

Reaction of poly A (Sigma, m.w. 139,000) with the three compounds of interest, ENU 10, BCNU 5 and CHNU 66 at 37°C and pH 7.0 followed by molecular weight analysis using sedimentation velocity on a Beckman analytical ultracentrifuge, resulted in the curves observed in Figure 37. The rate of RNA degradation parallels the rates of decomposition of the three nitrosoureas at pH 7.0

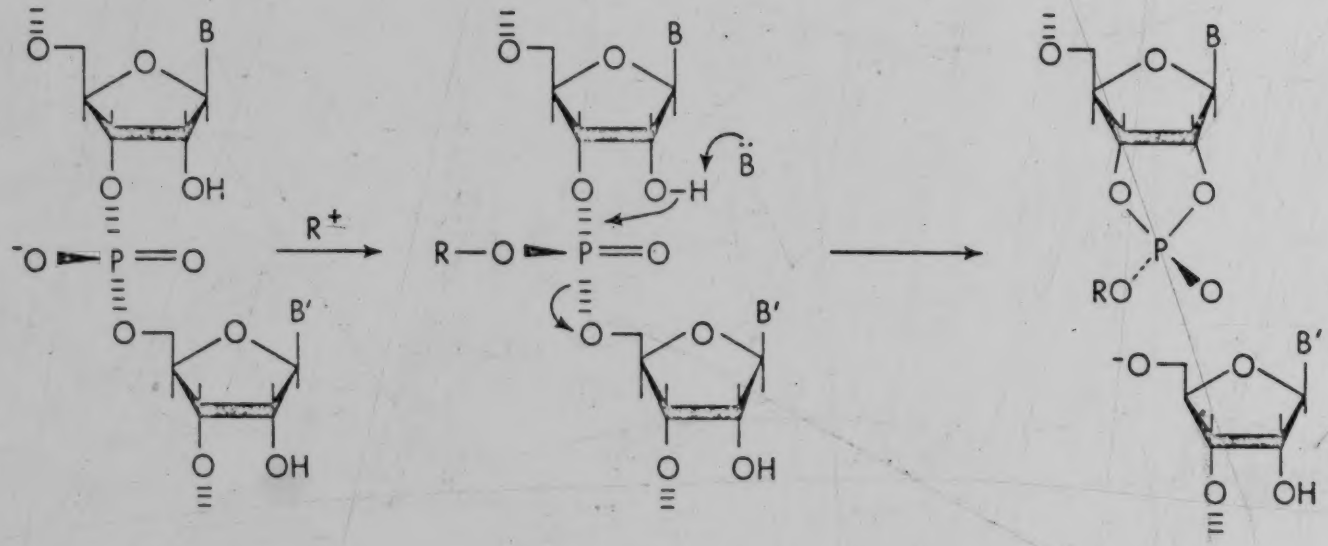


Figure 36. Hydrolysis of an RNA phosphotriester catalyzed by the 2′-hydroxyl group of the ribose.

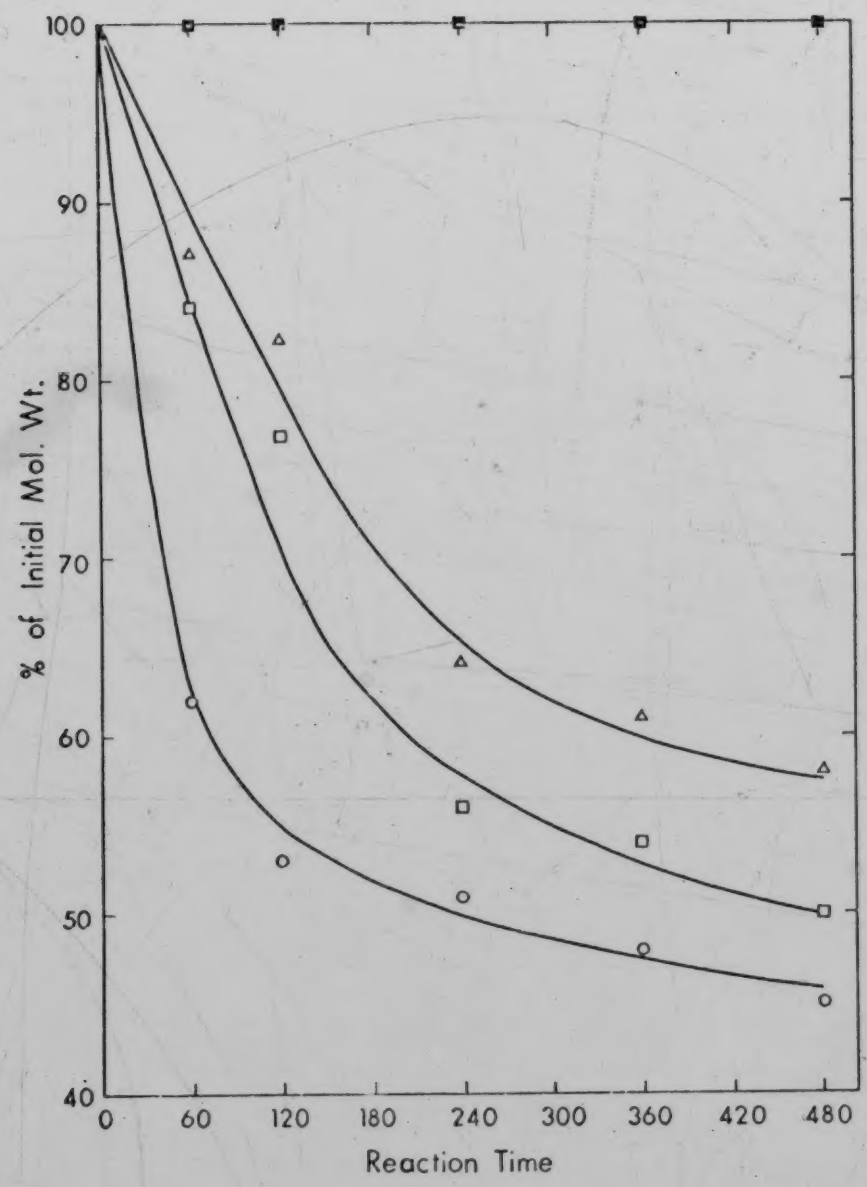


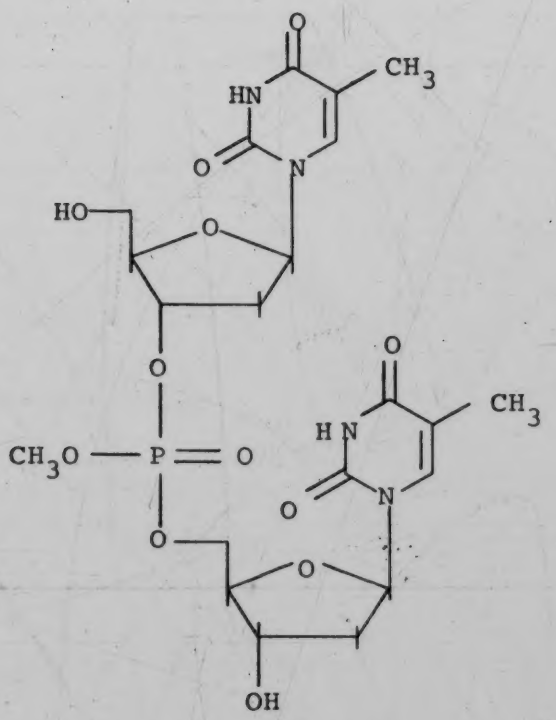
Figure 37. Decrease in poly A molecular weight during reaction of 150 mM drug with 4 mg/ml poly A (Sigma) in 0.05 M cacodylate buffer at 37°C. (O) ENU 10; (□) BCNU 5; (Δ) CHNU 66; (■) control, poly A (Sigma) pH 7.0 and 37°.

and 37°C. The half-lives under these conditions have been measured as ENU 10 16 min., BCNU 5 79 min., and CHNU 66 186 min. (see Chapter II). The extents of RNA degradation by the three compounds is approximately the same but whether or not this reflects similar extents of phosphate alkylation cannot be stated with certainty. To determine if the type I SSS observed for these compounds results from hydrolysis of the phosphotriesters formed, the nature and the fate of the triesters resulting from the three different alkylating agents was examined.

Lawley *et al.* have observed that the methyl phosphotriester of the thymidylyl(3'-5')thymidine dinucleotide 164 has a half-life of 2.3 h. in 0.1 N sodium hydroxide at 37°C.[162] Shooter[163] has reported the slow rates of alkaline hydrolysis of ethyl phosphotriesters in DNA while, as stated above, Verly and Bannon have observed methyl and ethyl DNA phosphotriesters are stable under neutral conditions.[33]

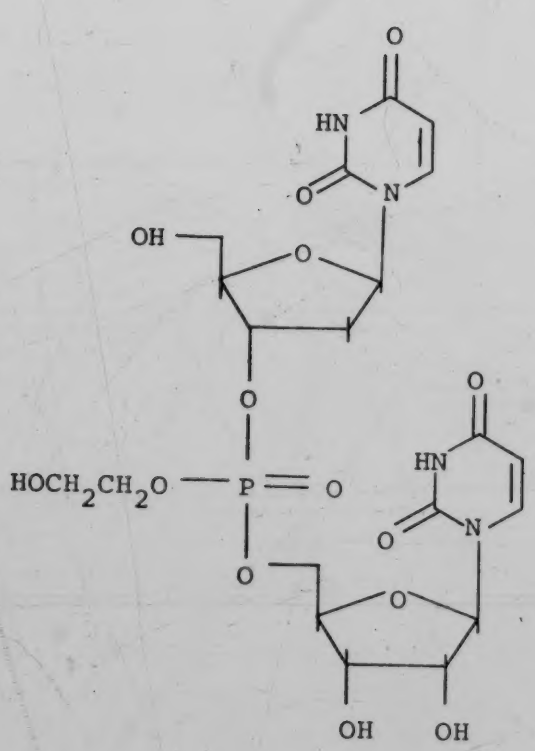
No work has been reported for the stability of chloroethyl phosphotriesters which will be discussed shortly.

Hydroxyethyl phosphotriesters of DNA have been reported to result in strand scission under neutral conditions.[167,168] However, there is some disagreement concerning DNA strand scission after hydroxyethylation.[158]



164

Mikhailov and Smrt have observed that the β-hydroxyethyl phosphotriester of the deoxyuridylyl-(3'-5')-uridine dinucleotide 165 prepared recently is stable at pH 7.5 and 40°C but will readily undergo base catalyzed hydrolysis in aqueous ammonia at 20°C to yield a mixture of nucleotide products.[169]



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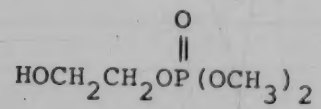
While the enthalpy of hydrolysis of β-hydroxyethyl dimethyl phosphate 166 has been reported to be only slightly greater than that observed for trimethyl phosphate 167,[170,171]



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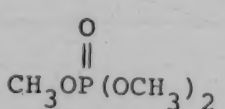


167

there have been observations which indicate that some β-hydroxyethyl phosphates hydrolyze very rapidly under alkaline conditions.[172,173]

To determine the stability of the DNA phosphotriesters 168, 169, 170, formed by ethyl, chloroethyl and hydroxyethyl alkylating agents (Fig. 38) under the alkaline assay conditions used to observe type I SSS, three model compounds were prepared. Triethyl phosphate 171, β-chloroethyl diethyl phosphate 172 and β-hydroxyethyl diethyl phosphate 173 (Fig. 39) were subjected to the alkaline conditions of the assay (pH 11.8, ambient temperature) and the extent of hydrolysis measured using gas-liquid chromatography.

Both triethyl phosphate 171 and β-chloroethyl diethyl phosphate 172 were stable under the high pH conditions with negligible hydrolysis after six hours at ambient temperature. A trace amount of ethanol could be identified in the hydrolysis mixture but this accounted for less than 5% of the volatiles for each compound (Fig. 39). However, in the case of the β-hydroxyethyl diethyl phosphate 173 the results were significantly different. An aliquot of the hydrolysis mixture was injected into the gas liquid chromatograph within 30 seconds after addition of the phosphate to the pH 11.8 solution. The chromatography indicated that the β-hydroxyethyl diethyl phosphate 173 had completely hydrolyzed. Ethanol accounted

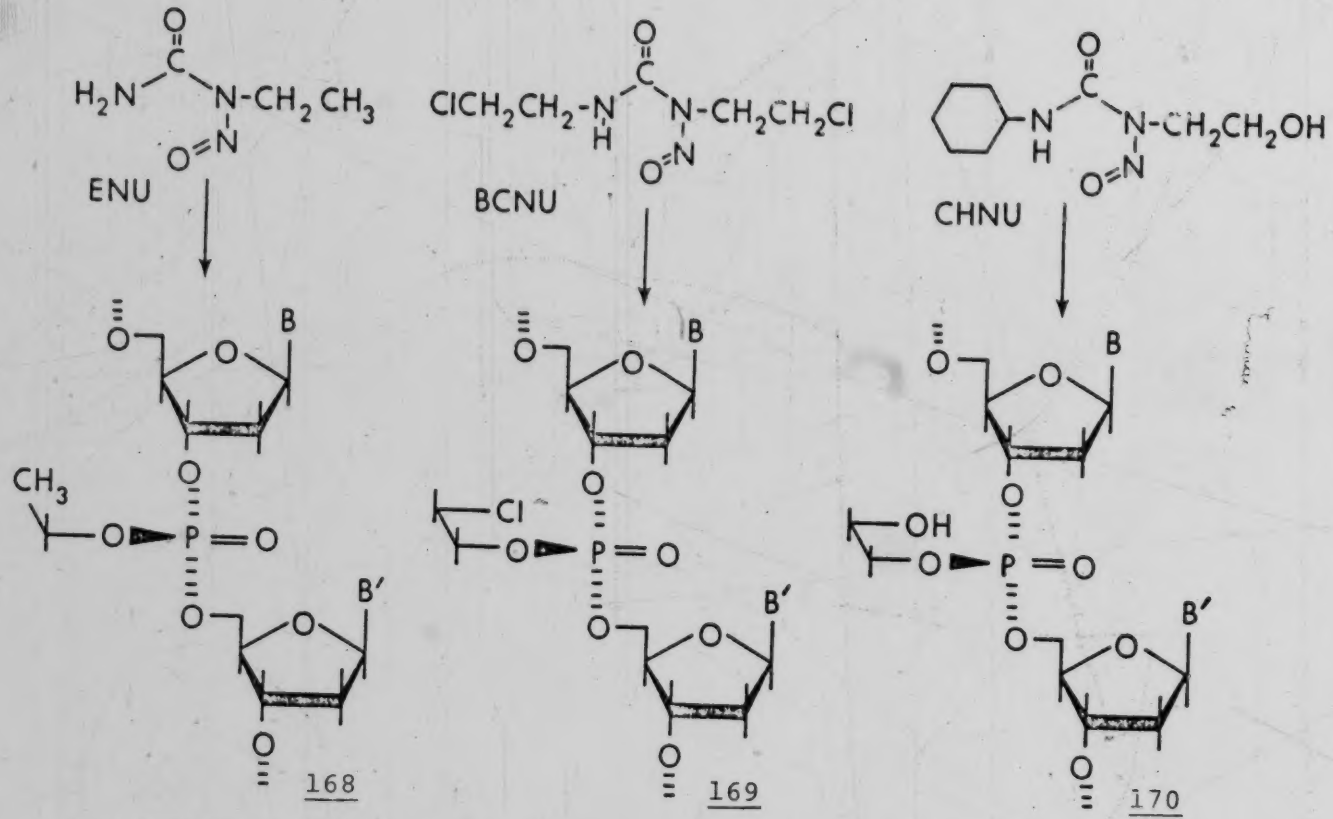


Figure 38. Phosphotriesters resulting from reaction of ENU 10, BCNU 5 and CHNU 66 with DNA.

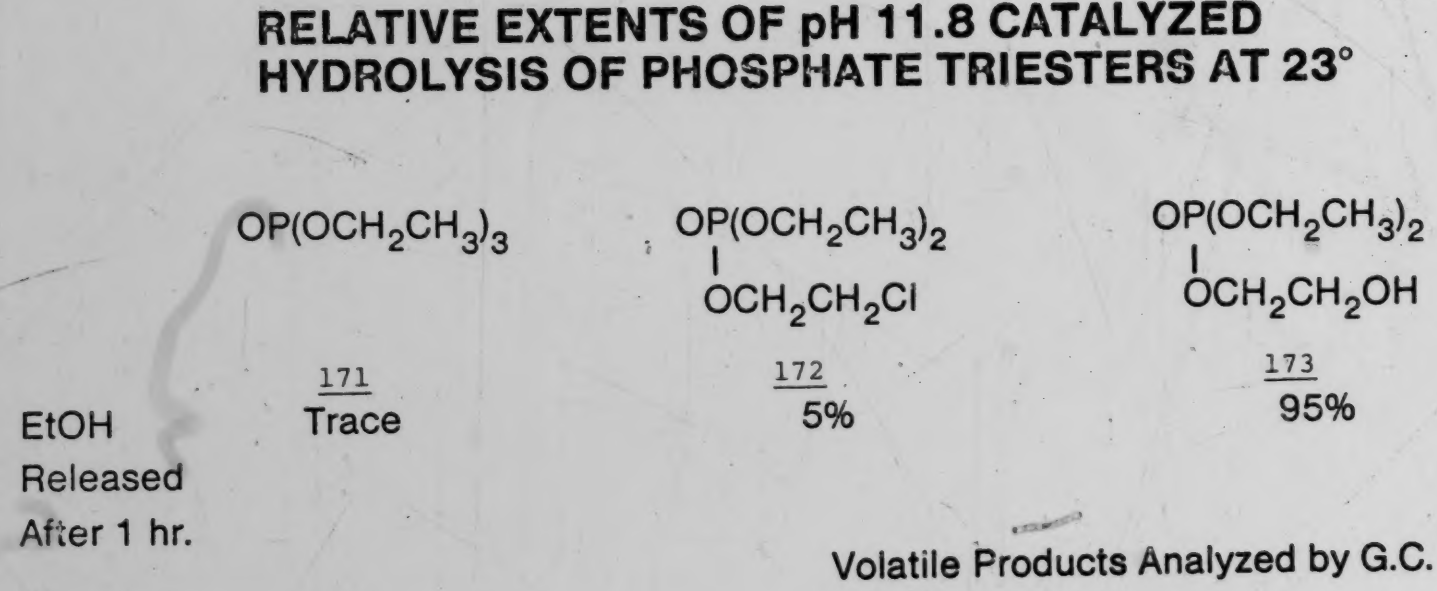
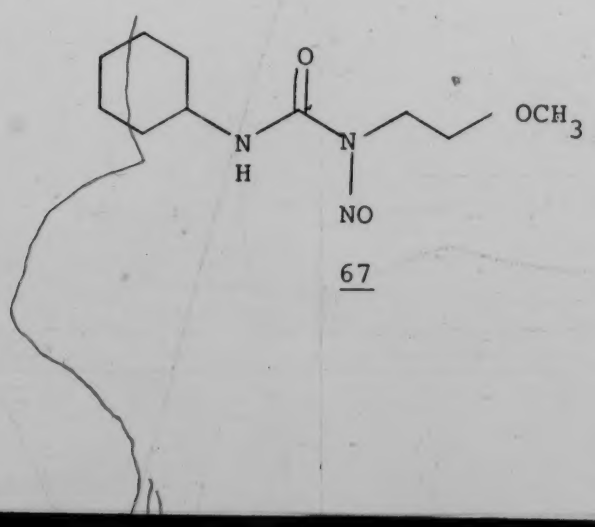


Figure 39. Extent of hydrolysis of model phosphotriesters as measured by released ethanol.

for 95% of the volatile products swept from the column within 20 minutes of sample injections (Fig. 39).

Whether or not the observed rapid hydrolysis for the β-hydroxyethyl derivative is the result of a concerted S_N2 mechanism,[174] an addition elimination mechanism[175] or, a cyclic pentacoordinate intermediate which has been suggested from some β-hydroxyethyl phosphodiesters,[176] as well as ribonucleotides,[177] is not within the scope of this study.

The extreme lability of this model β-hydroxyethyl phosphotriester 173 accounts for the rapid type I SSS observed for CHNU 66 (Fig. 1). While conversely, the stability of triethyl phosphate 171 to the alkaline assay conditions accounts for the very low type I SSS observed for ENU 10. While both compounds alkylate the internucleotide phosphate groups extensively, the fate of the triesters is significantly different in alkaline solution. Further evidence as to the necessity of the hydroxyl function can be obtained by comparing the results obtained for 3-cyclohexyl-1-(2-methoxyethyl)-1-nitrosourea 67. By



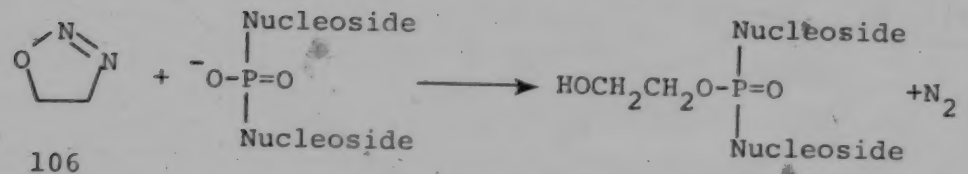
67

methylating the hydroxyl function, near total inhibition of type I SSS was observed (Fig. 29).

The stability of the β-chloroethyl diethyl phosphate 172 in the pH 11.8 solution does not agree with the observed significant type I SSS noted for chlorozotocin 86 and BCNU 5 (Fig. 29). It appeared that the most likely route to type I SSS by chloroethylating agents would involve some hydrolysis of the chlorine to produce the labile hydroxyethyl derivative. Previous work[81,102] has shown that hydroxyethylated bases can be isolated after treatment of synthetic polynucleotides with BCNU 5. To detect this possible pathway, a solution of β-chloroethyl diethyl phosphate 172, pH 7.2 was incubated at 37°C. At one hour intervals aliquots of this reaction mixture were transferred to a pH 11.8 buffered solution which was then chromatographed. No observed change in the concentration of β-chloroethyl diethyl phosphate 172 was observed in a three hour period. After the incubation, a sample of the reaction mixture at pH 7.2 was chromatographed. No β-hydroxyethyl diethyl phosphate 173 was detected.

While the model β-chloroethyl compound did not appear to follow the postulated hydrolytic pathway this does not preclude the possibility that some hydrolysis of the chlorine occurs during transport or decomposition of chloroethyl nitrosoureas. Clearly the isolation of hydroxyethylated bases by Ludlum *et al.*[81,102] after

treatment of polyribonucleotides with BCNU 5 suggests chloride hydrolysis at some stage of the reaction. The observation in Chapter III that no hydrolysis of chloroethylcytosine derivatives to hydroxyethylcytosine derivatives was detected also indicates that the necessary hydrolysis must occur prior to alkylation. A possible explanation for the production of hydroxyethyl phosphotriesters from chloroethylnitrosoureas involves reaction of the 1,2,3-oxadiazoline 106 suggested in Chapter II to result from chloroethylnitrosourea decomposition. Reaction of the DNA phosphodiester 174 with the 1,2,3-oxadiazoline 106 could result in the labile β-hydroxyethyl phosphotriester 175.



106 174 175

In support of this hypothesis, BFNU 26 is observed to produce significantly less type I SSS than BCNU 5 (Fig. 29). The greater strength of the carbon-fluorine

bond would inhibit either hydrolytic pathways or cyclization pathways to produce the 1,2,3,-oxadiazoline 106. In either case, less β-hydroxyethyl phosphotriester 175 and less type I SSS would be expected.

Conclusions

There is considerable evidence that the nitrosoureas react primarily so as to alkylate DNA and to form interstrand cross-links. These processes are also accompanied by single strand scission of the DNA. The present work indicates the latter process can occur by two distinct pathways (i) a relatively rapid reaction involving deoxyribosephosphate triesters and subsequent hydrolysis and (ii) base alkylation followed by a relatively slower depurination and hydrolysis of the apurinic site either enzymatically or by reaction with an amine. The isolation of β-hydroxyethyl substituted pyrimidine moieties from nitrosourea treated DNA and the observed efficient DNA scission by CHNU which contains a β-hydroxyethyl substituent suggested that the therapeutic properties of this class of nitrosoureas should be examined.

The effects of electrophiles from nitrosoureas on DNA is shown in Figure 40. Alkylation can occur on the purine or pyrimidine bases or the internucleotide phosphate linkages. Phosphate alkylation can result in rapid type I SSS while base alkylation followed by depurination

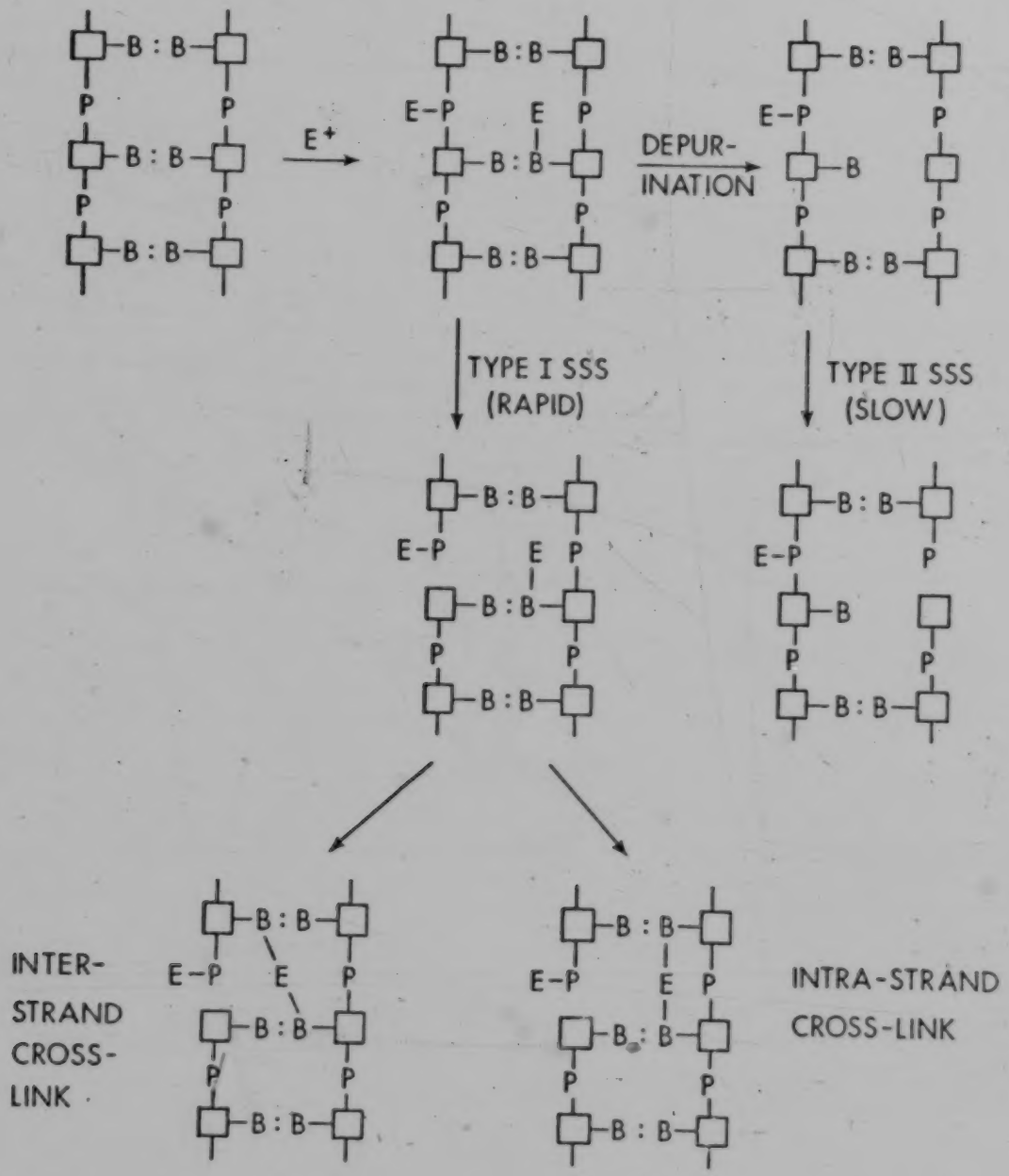


Figure 40. Effects of electrophiles resulting from 2-haloethylnitrosoureas on DNA.

(or depyrimidination) produces a slow type II SSS involving hydrolysis of labile apurinic sites. Base alkylation can also be followed by a second intermolecular or intramolecular alkylation. Intermolecular alkylation produces either intrastrand or interstrand DNA cross-links.

Experimental

Materials

Triethyl phosphate 171 was obtained from Aldrich and was redistilled (b.p. 93-95°/12 mm). PM2-CCC-DNA and calf thymus topoisomerase were gifts from Dr. A. Richard Morgan, Department of Biochemistry, University of Alberta. Endonuclease VI was isolated according to the method of Verly and Rassart.[155]

β-Chloroethyl Diethyl Phosphate 172.

This compound was prepared according to the method of Robinson[178] b.p. 136-137/12 mm (lit. 144-145/18 mm). Pmr (CCl_4) δ 1.3 (t, 6H, CH_3); 3.7 (t, 2H, CH_2); 3.9-4.4 (m, 6H, CH_2).

β-Hydroxyethyl Diethyl Phosphate 173.

Ethyl ethylene phosphate[179] was allowed to solvolyze overnight in absolute ethanol. After solvent removal the product β-hydroxyethyl diethyl phosphate 173 is distilled in near quantitative yield b.p. 97-99°C/10^{-4} mm.

Anal. Calcd. for $C_6H_{15}PO_5$ (m.w. + proton 199.0736, m.w. - proton 197.0579): C, 36.36; H, 7.64. Found (199.0740,

197.0583 mass spectrum): C, 36.16; H, 7.56. Pmr ($CDCl_3$) δ 1.38 (t, 6H, CH_3); 3.34 (s, 1H, exchangeable); 3.72-4.32 (m, 8H, CH_2). Ir ν_{max} (film) 3400 (OH); 1260 (P=O); 1030 (P-O) cm^{-1}.

Depurinated PM2-CCC-DNA.

To 400 µl of PM2-CCC-DNA 8.0 A_{260} was added 25 µl 1M sodium acetate buffer pH 3.05. The mixture was incubated at 37°C. 2 µl aliquots were withdrawn and added to the standard assay solution (which was 20 mM phosphate, pH 11.8, 0.4 mM EDTA, and 0.5 µg/ml of ethidium) the fluorescence was measured and compared to that obtained after heating at 96°C/3 min and followed by rapid cooling (see methods).

Under these conditions unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Depurinated PM2-CCC-DNA shows a decrease in fluorescence due to alkaline strand scission of the apurinic site in the assay medium. The ratio of the decrease in fluorescence (after the heating and cooling cycle) to that of the control is a measure of the extent of depurination. As long as the initial fluorescence reading remains constant, DNA degradation other than depurination is negligible. Typically a 90-120 minute incubation is necessary to introduce at least one apurinic site per molecule. After incubation, 50 µl of 1 M pH 7.2

phosphate buffer is added to quench the reaction. The solution of apurinic PM2-CCC-DNA may be stored for several days at 4°C.

Endonuclease Specific for Apurinic Sites of Escherichia coli (Endonuclease VI).

This enzyme was prepared for this study by Joan Forsythe, Department of Biochemistry, University of Alberta. Endonuclease VI was purified according to Verly and Rassart[155] from *E. coli* BATCC 11303, after the phosphocellulose chromatography the enzyme was stored in 0.15 M NaCl, 0.04 M sodium phosphate pH 6.5 with an equal volume of glycerol and kept at -20°. For the experiments, this preparation was diluted with a suitable buffer.

Assay for Endonuclease VI Activity.

The basis of the assay is that the enzyme cleaves apurinic PM2-CCC-DNA and thereby converts it to linear DNA which results in a change in ethidium fluorescence both before and after heat denaturation when measured at pH 8.0. The reaction solution consisted of apurinic PM2-DNA 1.0 A_{260} units in potassium phosphate buffer pH 8.0. A 10 λ aliquot of the enzyme was added and the reaction solution incubated at 37° for 15 min and the fluorescence of the resulting PM2-OC-DNA read using the standard pH 8 ethidium assay. Conversion of PM2-CCC-DNA to PM2-CC-DNA by the endonuclease VI results in a

characteristic 30% increase in fluorescence as a result of the release of topological constraints. After heat denaturation at 96°/3 min, when the PM2-OC-DNA is converted into single strands, then rapid cooling to 23° the fluorescence was read again. An active endonuclease VI fraction is revealed by loss of fluorescence after heat denaturation. The control for the assay consisted of a similar reaction substituting native PM2-CCC-DNA.

Methods

Ethidium Fluorescence Assay for Type I SSS of DNA.

The fluorometric methods using ethidium bromide have been described in Chapter III. The conversion of PM2-CCC-DNA to PM2-OC-DNA results in a 30% increase in fluorescence in the pH 11.8 ethidium assay solution (which was 20 mM potassium phosphate, pH 11.8, 0.4 mM EDTA and 0.5 µg/ml of ethidium bromide) owing to release of topological constraints.

A 300 µl sample containing PM2-CCC-DNA 1.0 A_{260}, 50 mM sodium cacodylate buffer pH 7.0 and 400 mM NaCl was incubated at 37°C with the topoisomerase. The fluorescence was monitored by transferring 20 µl aliquots into 2 ml of the pH 11.8 assay solution. When a 25-30% decrease in fluorescence had been observed (typically requiring a 30 min incubation), a 5 mM concentration of the desired drug was introduced and the fluorescence again monitored

using 20 µl aliquots in 2 ml of the pH 11.8 assay solution. Readings must be taken immediately after addition of the aliquot so that apurinic site hydrolysis does not contribute to the observation of type I SSS.

Ethidium Fluorescence Assay for Type II SSS of DNA.

After the fluorescence reading had been taken to determine type I SSS and pH 11.8 assay solution containing the 20 µl aliquot of reaction mixture was incubated at 37°C. At designated times, the solution was reequilibrated to 22°C for the fluorescence reading.

Detection of Apurinic Sites.

A 300 µl solution containing 5 mM drug, 50 mM sodium cacodylate pH 7.0 and relaxed PM2-CCC-DNA 1.0 A_{260} was allowed to react for 120 min while monitoring for type I SSS. 20 µl of the apurinic endonuclease solution was then added (the amount was determined by previous experiments with low pH depurinated PM2-CCC-DNA). The fluorescence was then monitored as described in part 1 of Methods section. The percent of fluorescence increase with respect to the fluorescence at time 0 min was corrected for dilution by the enzyme solution.

Reaction of Apurinic DNA with Amines.

A 200 µl solution containing apurinic PM2-CCC-DNA 1.0 A_{260} 50 mM potassium phosphate pH 7.2 and 5 mM of

of the appropriate amine was incubated at 37°C. 20 µl aliquots were withdrawn and added to the pH 11.8 assay solution and the fluorescence reading immediately taken. A control solution was monitored which contained the apurinic PM2-CCC-DNA in a pH 7.2 buffer at 37°C.

Detection of Phosphate Alkylation by RNA Degradation.

A 140 µl solution containing 4 mg/ml Poly A (Sigma m.w. 139,000), 150 mM sodium cacodylate buffer pH 7.0 and 150 mM of the desired nitrosourea was incubated from 1-8 h. The reactions were quenched in ice and dialyzed against 50 mM potassium phosphate pH 7.2, 100 mM NaCl, 1 mM EDTA in triply distilled water at 4°C for 36 h. The dialysate was then diluted with the dialysis solution to 1.0 A_{260} and the sedimentation velocity determined on a Beckman Analytical Ultracentrifuge.

Phosphotriester Hydrolysis.

Gas-liquid chromatographic analysis of the triesters was performed on a Hewlett-Packard model 5830 A temperature programmable research chromatograph equipped with a flame ionization detector. To a 1 ml solution containing 200 mM potassium phosphate pH 11.8 and 0.5% dioxane as an internal standard was added the appropriate triester to a concentration of 20 mM. A 1 µl aliquot was immediately injected, after thorough mixing, onto a 6 ft stainless steel column containing a support of 10% polyphenyl ether

on chromosorb W. The column temperature was maintained at 150°C for 4 min at which time it was heated at 20°C/ min until a temperature of 200°C had been reached. This temperature was maintained for 20 min or until all volatiles had been swept from the column. Additional 1 µl aliquots were taken from the solutions during the next 6 h. The following retention times were observed: ethanol 0.9 min, dioxane 1.9 min, triethyl phosphate 7.8 min, β-chloroethyl diethyl phosphate 14.4 min and β-hydroxyethyl diethyl phosphate 16.7 min.

Attempted Conversion of β-Chloroethyl Diethyl Phosphate to β-Hydroxyethyl Diethyl Phosphate.

A 200 µl solution containing 10% dioxane, 100 mM potassium phosphate pH 7.2 and 25 mM β-chloroethyl diethyl phosphate was incubated at 37°C. At 1 hour intervals a 10 µl aliquot was transferred to a 250 µl solution containing 200 mM potassium phosphate pH 11.8 and a 1 µl aliquot of this solution was injected onto the polyphenyl ether column as described above. After a 3 h incubation, a 1 µl sample of the pH 7.2 reaction mixture was injected onto the column.

CHAPTER FIVE

NOVEL NITROSOUREAS AND RELATED COMPOUNDS AND THEIR REACTIONS WITH DNA

Introduction

The previous three chapters have examined various aspects of the chemistry of the nitrosoureas including aqueous decomposition, alkylation of DNA (including interstrand cross-linking) and DNA single strand scission. The results of this investigation suggest that significant DNA cross-linking by nitrosoureas requires the generation of a chloroethyl alkylating agent, possibly the cyclic chloronium ion, upon aqueous decomposition. Chloroethylation of an appropriate base in DNA is followed by labilization of the carbon-chlorine bond and a second alkylation to complete the cross-link.

The correlation between ability to produce DNA interstrand cross-links and activity in the leukemia L1210 test system (see Table 13, Chapter III) has prompted an attempted rational design of nitrosoureas and similar compounds incorporating five design features.

(i) The modification of nitrosoureas and/or similar structures to increase the rate of decomposition and production of the chloroethyl alkylating agent may increase the rate and extent of cross-linking.

(ii) The modification of the carbon bearing the halogen to produce compounds with better leaving groups

may increase the efficiency of the second alkylation necessary for the cross-link and lead to enhanced therapeutic effects.

(iii) The modification of nitrosoureas to nitrosothioureas will result in isothiocyanates in place of isocyanates upon decomposition under physiological conditions. Isothiocyanates being less reactive than their oxygen counterparts may show reduced carbamoylating activity and toxicity effects.

(iv) The design of compounds which will produce chloroethyl alkylating species from sources other than nitrosoureas may result in comparable DNA cross-linking and therapeutic effects.

(v) The modification of nitrosoureas to produce alkylating agents other than chloroethyl alkylating species may result in enhanced interstrand cross-linking and therapeutic effects.

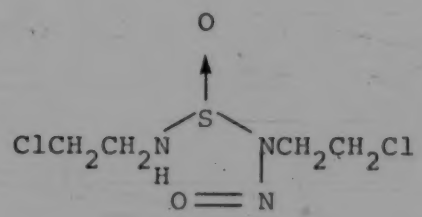
Modification of the substituent which remains attached to the isocyanate portion of the molecule has been carried out in a number of previous studies.[2,3,15] Therefore, this aspect of nitrosourea modification was not explored in the present investigation.

(i) Studies Related to the Rate of Production of Chloroethyl Alkylating Species.

The previous work described in Chapter III has indicated that BCNU 5 exhibits significant DNA interstrand

cross-linking which maximizes after 6-8 h. BCNU 5 in a pH 7.2 aqueous buffered solution at 37°C has a half-life of 79 min. The rate of aqueous decomposition could be increased significantly by preparing the unsubstituted derivative 1-(2-chloroethyl)-1-nitrosourea (CNU) 3 which under the same conditions has a half-life of 8 min. CNU 3 produces interstrand cross-links comparable to that of BCNU 5 which maximize in 4 h. The observation that an increase in decomposition rate by a factor of 10 only increases the rate of cross-linking by approximately a factor of 2 supports the hypothesis that it is not the decomposition and initial alkylation but rather the rate of the second alkylation by the intermediate chloroethylated base which determines the rate of cross-linking.

A second approach to increase the rate of production of the chloroethyl alkylating species was attempted by preparing the sulfoxide analogue of BCNU 5. Addition of aziridine to thionyl chloride followed by aqueous nitrosation resulted in a compound which appeared to be the N,N'-bis(3-chloro-1-azapropyl)-N-nitrososulfoxide 176,



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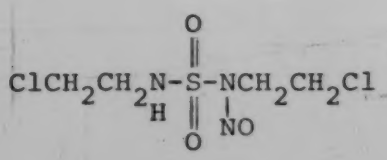
however, it proved to be too unstable to purify. By a similar procedure using aziridine and sulfuryl chloride followed by anhydrous nitrosation an attempt was made to prepare the 1,3-bis(2-chloroethyl)-1-nitrosodisulfonamide 177. Nitrosation of the 1,3-bis(2-chloroethyl)di-



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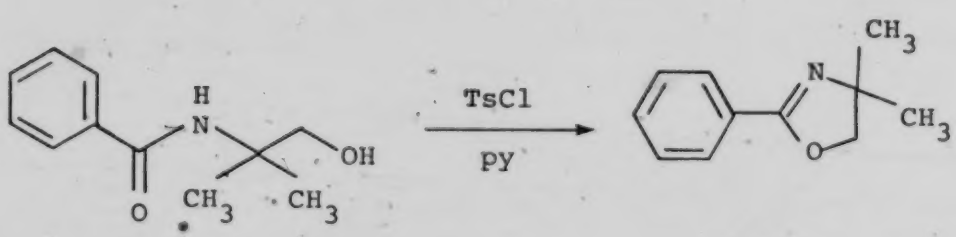
sulfonamide at -30°C produced an extremely unstable compound.

(ii) Studies Related to Increasing the Efficiency of the Second Alkylation of the Cross-link.

While the second alkylation necessary to complete the cross-link occurs at the carbon bearing the chlorine, increasing its ability to act as a leaving group does not appear to increase the efficiency of cross-linking. Chloroethylation of nitrogen atoms in the DNA molecule would produce intermediate compounds which resemble nitrogen mustards. The ability of these intermediates to result in a second alkylation forming the interstrand cross-link should parallel the alkylating ability of nitrogen mustards.

The relative alkylating ability of chloro, bromo and iodo nitrogen mustards has been previously reported[180] using a p-nitrobenzylpyridine test for alkylating ability. The relative values for Cl, Br and I phenyl nitrogen mustards are 1.0, 18.6 and 20.2, respectively. However, the extent of cross-linking produced by a chloroethyl, bromoethyl and iodoethyl nitrosoureas is 43%, 8% and <2%, respectively.

Further modification of the ethyl substituent to produce p-toluenesulfonate esters was abandoned since Hansen and Neilson[181] have reported that a similar benzamide derivative 178 undergoes cyclization to form an oxazoline 179 at room temperature.



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The parallel between ability to act as a leaving group and ability to produce DNA interstrand cross-links appears to break down. As the group attached to the carbon which normally bears the chlorine atom in 2-chloroethyl nitrosoureas increases in leaving ability, competitive

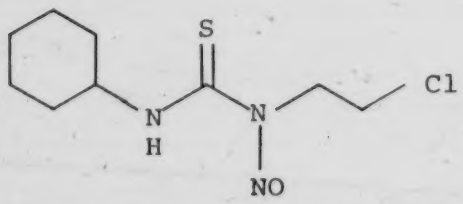
decomposition pathways involving the substituted 2-imino-N-nitroso-oxazolidinone and/or the oxadiazoline and/or hydride migration result in less of the desired 2-substituted ethyl alkylating agent.

A more productive approach to increase the efficiency of the second alkylation in the formation of a cross-link is detailed in part (v) of this investigation.

(iii) Studies Related to Nitrosothioureas.

Carbamoylation by the isocyanates generated from the decomposition of nitrosoureas has been related[5,8-10] to toxicity effects. In an attempt to inhibit the carbamoylating properties without major structure modification the N-nitrosothiourea analogue of CCNU 6 was prepared. Isothiocyanates are less reactive[182] than isocyanates toward alcohols and amines and should therefore have lower carbamoylating activities when produced *in vivo* from the decomposition of nitrosothioureas.

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosothiourea 180, prepared in an analogous manner to CCNU 6, exhibits



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significant DNA cross-linking at pH 7.2 and 37°C (54% cross-linked λ-DNA in 6 h) in comparison to CCNU 6 (43% cross-linked λ-DNA in 6 h). Preliminary *in vivo* screening results obtained from the National Institutes of Health, Bethesda, Maryland indicate that the thio derivative of CCNU 180 has a % (T/C) value of 523 against the leukemia L1210 test system. Under comparable conditions, CCNU 6 has a % (T/C) of 307.[183]

The high activity exhibited by this derivative indicates that additional compounds in this group should be prepared for further study and antileukemic testing.

(iv) Studies Related to Chloroethyl Alkylating Agents.

Of the nitrosoureas investigated in this study, the 2-chloroethyl derivatives decompose most efficiently to produce the desired 2-substituted ethyl alkylating agent necessary for cross-linking (see Chapter II). The chlorine atom is not an exceptional leaving group such that competing decomposition pathways involving oxadiazolines, imino-N-nitrosooxazolidinones or hydride migrations which result in species other than the desired 2-chloroethyl alkylating agent are favorable. However, the ability of the chlorine to act as a leaving group is such, that after chloroethylation of an appropriate base, labilization of the chlorine to substitution and production of interstrand cross-links occurs.

Therefore, chloroethyl akylating agents from a number of sources were examined. Initial experiments involved the chloroethyl species $ClCH_2CH_2$-L with leaving groups L other than the diazohydroxide or diazonium ion which result from the nitrosoureas. A series of compounds was prepared or purchased which included: 2-chloroethyltrifluoromethanesulfonate 181, 2-chloroethyl-p-nitrobenzenesulfonate 182, 2-chloroethyl-p-toluenesulfonate 183, 2-chloroethylmethanesulfonate 184 and tris-(2-chloroethyl)phosphate 185. The alkylating ability of these

$ClCH_2CH_2$-L

181 -L = $-OSO_2CF_3$

182 -L = $-OSO_2C_6H_4NO_2$

183 -L = $-OSO_2C_6H_4CH_3$

184 -L = $-OSO_2CH_3$

185 -L = $-OP{=}O(OCH_2CH_2Cl)_2$

derivatives in comparison to selected nitrosoureas is listed in Table 14. Only the trifluoromethanesulfonate 181 derivative, other than the nitrosourea, exhibited significant alkylation of PM2-CCC-DNA. This derivative also resulted in 11% cross-linked λ-DNA after a reaction time of 6 h (by comparison CNU 3 cross-links 37% λ-DNA in 6 h). The observation that the remaining sulfonate esters exhibit no alkylating ability is unclear since

Table 14

Leaving Group Ability Nucleophilic Displacements -Decreasing Order[184]	Relative Rate of PM2-DNA Alkylation (% Alkylation by 5 mM drug in 180 min)	
	Compound	% Alkylation
$R\text{-}\overset{+}{N} \equiv N$	1-(2-Chloroethyl)-1-nitrosourea 3	70
	Ethyl nitrosourea 10	53
$ROSO_2CF_3$	2-Chloroethyl trifluoromethane sulfonate 181	40
$ROSO_2C_6H_4pNO_2$	2-Chloroethyl p-nitrobenzene sulfonate 182	0
$ROSO_2C_6H_4pCH_3$	2-Chloroethyl p-toluenesulfonate 183	0
$ROSO_2CH_3$	2-Chloroethyl methanesulfonate 184	0
$ROP(OR')_2$	Tris-(2-chloroethyl)phosphate 185	0

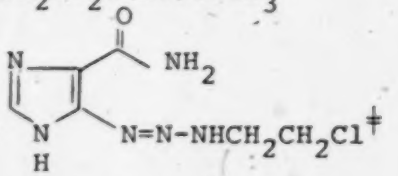
similar methyl and ethyl sulfonate esters alkylate DNA.[95]

Reutov *et al.*[185,186] have reported that the acetolysis of 2-chloroethyl p-nitrobenzenesulfonate 182 at elevated temperatures results in products which suggest some participation by a cyclic chloronium ion. While this observation indicates that the nosylate 182 generates a reactive species which could lead to DNA cross-linking, no such activity was observed at pH 7.2 and 37°C.

As reported in Chapter III, chloroethylnitrosoureas appear to alkylate by an S_N1 or low activation S_N2 reaction. Therefore, a second series of compounds was prepared which could result in 2-chloroethylcarbonium ions, 2-chloroethyldiazonium ions or similar alkylating species. A series of compounds including: 1-(2-chloroethyl)-3-nitro-1-nitrosoguanidine 186,[187] N-(2-chloroethyl)-N-nitrosoacetamide 187, 5-[3-(2-chloroethyl)triazenyl]imidazole-4-carboxamide 188,[188] ethyl N-(2-chloroethyl)-N-nitrosocarbamate 189 and N-(2-chloroethyl)-N-nitroso-p-toluenesulfonamide 190,[2] (Table 15) were assayed for their aqueous stability and ability to produce DNA interstrand cross-links. Polarographic analysis as described in Chapter II was used to determine the stabilities of these derivatives at 37°C in aqueous solution buffered to pH 7.1 (Table 15). The ability to produce DNA interstrand cross-links was assayed and compared to known antileukemic activities (Table 16).

Table 15

Polarographic Behavior of Related Nitroso Compounds

#	Compound	$E_{1/2}$, 1	$E_{1/2}$, 2	$t_{1/2}$(min)
3	$ClCH_2CH_2N(NO)CONH_2$	-0.752	-1.010	8 ± 4
186	$ClCH_2CH_2N(NO)C(=NH)NHNO_2$*	-0.543	-1.142	stable
187	$ClCH_2CH_2N(NO)COCH_3$*	-0.652	-1.152	51 ± 5
188		-0.998	-	5 ± 2
189	$ClCH_2CH_2N(NO)CO_2CH_2CH_3$	-0.653	-1.073	81 ± 8
190	$ClCH_2CH_2N(NO)SO_2C_6H_4CH_3$*	-0.038	-1.042	stable

*5% ethanol. ‡5% dimethylsulfoxide

Table 16

#	Compound	% Interstrand Cross-linking at 6 h	Activity Against Leukemia L1210 %(T/C)/Ref.
3	1-(2-chloroethyl)-1-nitrosourea	37	263/2
186	1-(2-chloroethyl)-3-nitro-1-nitrosoguanidine	0	300/189
187	N-(2-chloroethyl)-N-nitrosoacetamide	44	-
188	5-[3-(2-chloroethyl)triazenyl]imidazole-4-carboxamide	33	226/188
189	Ethyl N-(2-chloroethyl)-N-nitrosocarbamate	12	140/2
190	N-(2-chloroethyl)-N-nitroso-p-toluenesulfonamide	0	117/2

Both the nitroguanidine 186 and p-toluenesulfonamide 190 derivatives show no ability to produce DNA cross-links which is in agreement with their observed stability in the aqueous buffer. Metabolic activation may be necessary to initiate decomposition by these two derivatives *in vivo* and result in the observed activities. Nevertheless a significant correlation between ability to produce DNA interstrand cross-links *in vitro* and activity against the leukemia L1210 test system can be observed in Table 16. These results encouraged us to modify a derivative which exhibits low activity and a low extent of cross-linking in an attempt to increase its cross-linking and possibly its activity against leukemia L1210. The derivative chosen for modification was ethyl N-(2-chloroethyl)-N-nitrosocarbamate 189.

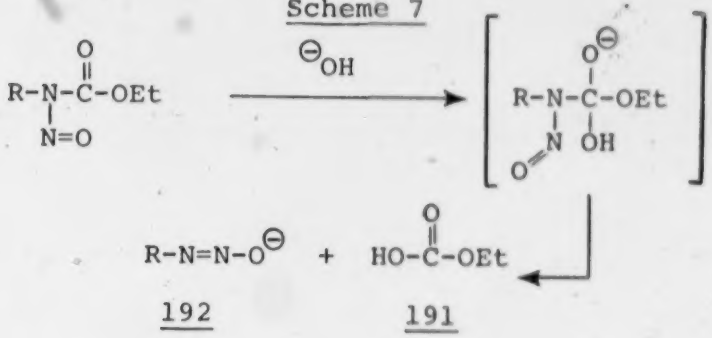
March has suggested[190] that the first step in the decomposition of similar nitrosocarbamates to produce diazoalkanes is a 1,3 nitrogen-to-oxygen rearrangement (Scheme 6). Nitrosocarbamate decomposition is base

Scheme 6

$$R\text{-}N(NO)CO_2Et \longrightarrow R\text{-}N{=}N\text{-}OCO_2Et$$

catalyzed which Smith[191] has suggested involves a nucleophilic attack by hydroxide ion at the carbonyl liberating a carbonic ester 191 and a diazotate 192 (Scheme 7).

Scheme 7



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While a slow 1,3 nitrogen-to-oxygen shift may explain the decomposition of ethyl N-(2-chloroethyl)-N-nitroso-carbamate 189 under physiological conditions, increasing the chance of nucleophilic attack at the carbonyl in a pH 7.2 buffered solution should increase the rate of decomposition and subsequent cross-linking.

To increase the nucleophilic character of the reaction mixture, DNA interstrand cross-linking for ethyl N-(2-chloroethyl)-N-nitrosocarbamate 189 was measured in the presence of excess 2-mercaptoethanol or dithio-threitol (Table 17). In both experiments it was conceivable that the thiol compound would compete favorably with the DNA for the chloroethyl alkylating moiety. However, in both experiments as observed in Table 17 the extent of DNA cross-linking increased significantly. Encouraged by these initial experiments a compound was designed which might *in vivo* result in similar nucleophilic activation of the nitrosocarbamate to produce the desired alkylating agents. The desired modified

Table 17

#	Compound	% Interstrand Cross-linking at 6 h
189	5 mM Ethyl N-(2-chloroethyl)-N-nitrosocarbamate	12
	+15 mM 2-mercaptoethanol	24
	+15 mM Dithiothreitol	28
194	2.5 mM 3,3'-Bis[N-(2-chloroethyl)-N-nitrosocarbamoyl]propyldisulfide	8
	+15 mM Dithiothreitol	25
	+25 mM Sodium Dithionite	21

nitrosocarbamate is shown in Figure 41. Reaction of bis(3-hydroxypropyl)disulfide with 2-chloroethylisocyanate produces the bis carbamate 193. Anhydrous nitrosation with N_2O_4 results in 3,3'-bis[N-(2-chloroethyl)-N-nitrosocarbamoyl]propyldisulfide 194. Reduction of the disulfide 194 *in situ* could produce the thiol derivative 195 which can interact intramolecularly or intermolecularly with the carbamate carbonyl. Intramolecular attack would result in the intermediate 196 shown in Figure 41. Proton transfer in the intermediate yields 2-chloroethyl diazohydroxide 197 and 1,3-oxathiane-2-one 198.

The results of the fluorometric assay for DNA interstrand cross-linking by this compound is shown in Table 17. The disulfide 194 is notably insoluble in aqueous solution. A 20% acetonitrile/water mixture still resulted in a cloudy solution. The low value for cross-linking after 6 h of reaction may in part be due to the low aqueous solubility. However, after 24 h of reaction, the reaction solution had cleared. The extent of cross-linking increased only to 15%. Conversely, addition of either dithiothreitol or sodium dithionite to the aqueous suspension of the disulfide resulted in a clear solution within 30 min, presumably due to the greater solubility of the thiol derivative 195. Both reducing agents result in enhanced cross-linking as compared to the parent disulfide (Table 17). The slightly greater value

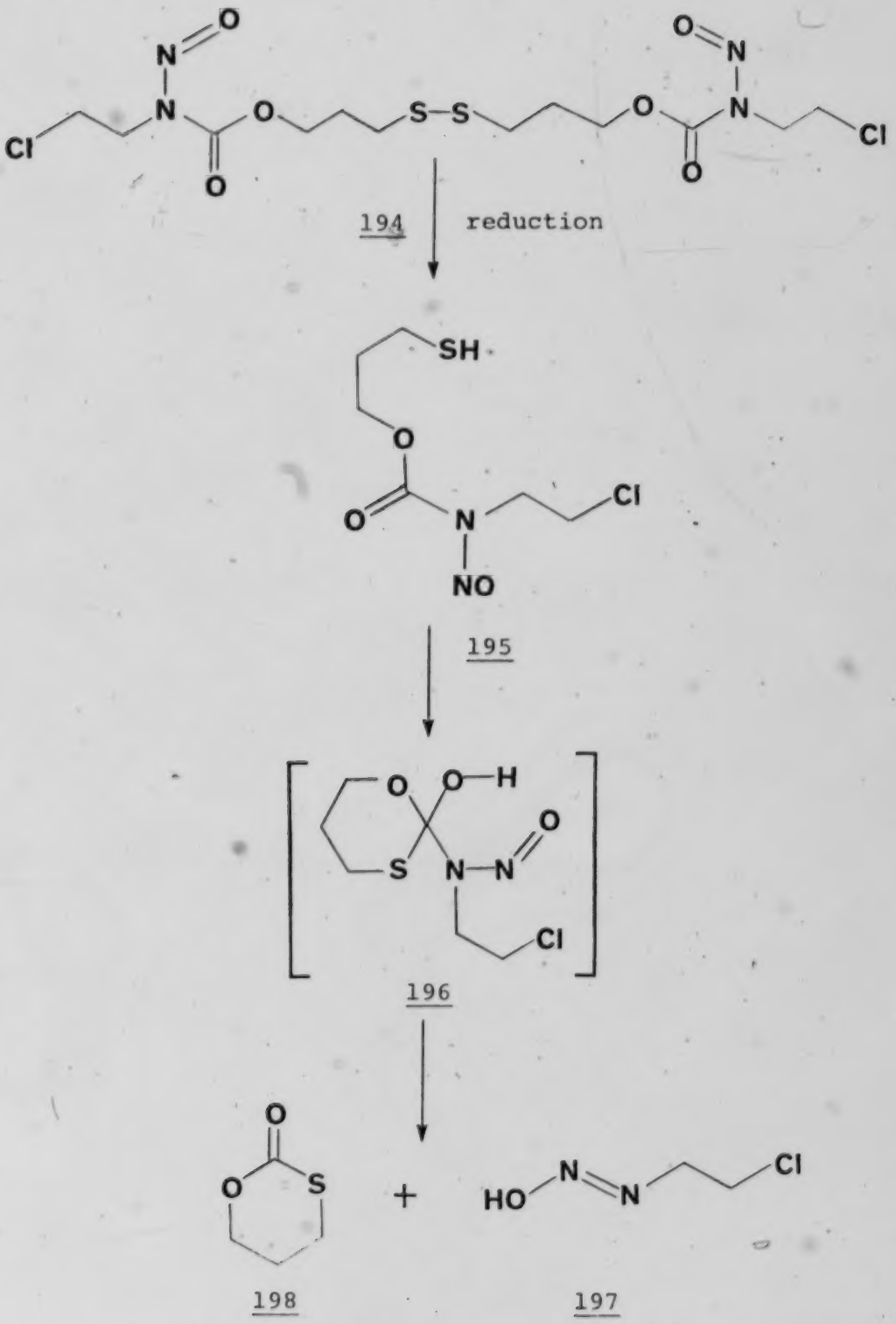


Figure 41. Proposed decomposition pathway following reduction of 3,3'-bis[N-(2-chloroethyl)-N-nitrosocarbamoyl]-propyldisulfide 194.

observed with dithothreitol may reflect the ability of this reducing agent to initiate a direct nucleophilic attack at the nitrosocarbamate carbonyl as well as to result in reduction of the disulfide linkage.

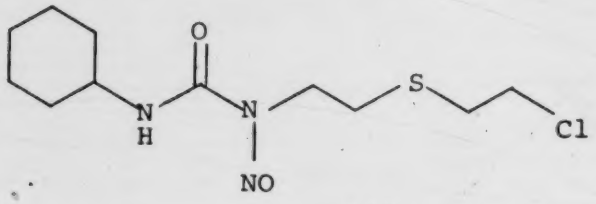
To confirm that some of the reduced disulfide undergoes intramolecular cyclization during the release of the chloroethyl alkylating agent an attempt was made to isolate the 1,3-oxathiane-2-one. A solution of 3,3'-bis-[N-(2-chloroethyl)-N-nitrosocarbamoyl]propyldisulfide 194 was allowed to decompose in a 200 mM pH 7.2 buffer solution at 37°C in the presence of sodium dithionite. Extraction of the aqueous solution with ether resulted in a mixture of compounds. High resolution mass spectral analysis indicated a molecular formula corresponding to the 1,3-oxathiane-2-one 198 (see Materials section).

(v) Studies Related to Nitrosoureas Which Produce Modified Alkylating Agents.

With the discovery that 2-chloroethylnitrosoureas exhibited high activity in the leukemia L1210 test system a wide variety of derivatives have been synthesized for biological testing. Most of the modifications of the alkylating portion of the molecule have already been outlined in Chapter III. Replacement of the chlorine atom by other halogens as well as chain lengthening or chain branching has generally resulted in a lowering or loss of activity. Therefore, most of the work in this

area has been concentrated on the modification of the substituent which results in the isocyanate upon decomposition. Encouraged by the correlation between the extent of DNA interstrand cross-linking and leukemia L1210 activity observed in Table 13 and Table 16, an additional attempt was made to modify the alkylating portion of the molecule to increase the extent of DNA cross-linking.

The design of this new compound is based upon information obtained as a result of this study. More specifically, the synthesis of this modified nitrosourea incorporated four design features. (i) The compound should inhibit decomposition pathways involving either the nitrosooxazolidinone or oxadiazoline thus more efficiently producing a cross-linking species. (ii) The intermediate carbonium ion should be stabilized in a manner similar to that occurring with the cyclic chloronium ion generated from chloroethylnitrosoureas. (iii) Substitution of the halide necessary for the second alkylation to complete the cross-link should be activated by the drug itself so that cross-linking will not be as dependent on the initial site of alkylation. (iv) The distance between the two alkylation sites should be greater than the restrictive two carbon link provided by chloroethylnitrosoureas. The compound synthesized based on these design features was 1-{2-[2-chloroethyl)-thio]ethyl}-3-cyclohexyl-1-nitrosourea 199. Decomposition,



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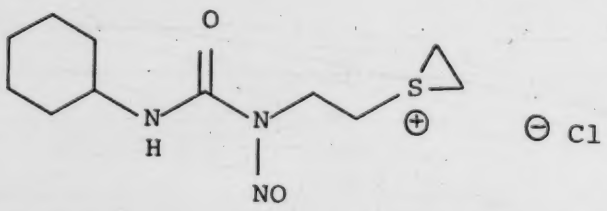
alkylation and cross-linking can be envisaged to occur as outlined in Figure 42. Initial DNA alkylation can occur by S_N2 reaction with the diazohydroxide 200 or by alkylation of the sulfonium ion 201 produced by S_N1 elimination of nitrogen and hydroxide. The second alkylation results from the reactive sulfur half-mustard 202 presumably through the sulfonium ion 203.

Conversely, due to labilization of the carbon-chlorine bond in the formation of the sulfonium ion 204



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the initial akylation may result from the sulfur half-mustard protion of the molecule followed by subsequent decomposition and alkylation by the nitrosourea.

This nitrosourea derivative produces 90% cross-linked λ-DNA in 10 min at pH 7.2 and 37°C. Recent *in vivo* testing of this compound resulted in a % (T/C) value of 194.

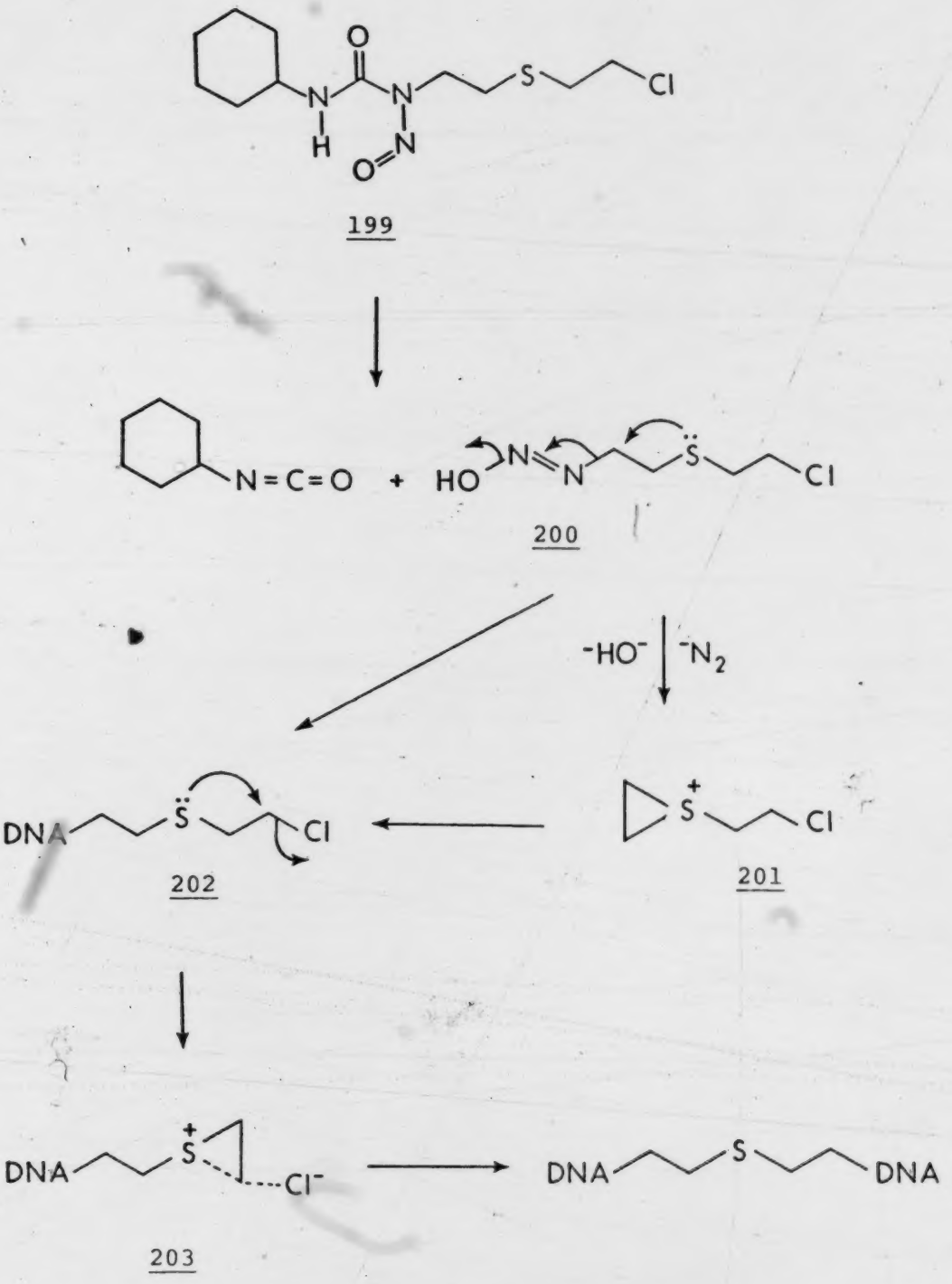
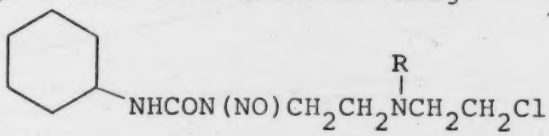


Figure 42. Proposed pathway for decomposition and DNA inter-strand cross-linking by 1-[2-[(2-chloroethyl)-thio]ethyl]-3-cyclohexyl-1-nitrosourea 199.

The significant activity displayed as a result of this modification encouraged the synthesis of similar derivatives. To date only the oxygen analogue, 1-[2-(chloroethyoxy)ethyl]-3-cyclohexyl-1-nitrosourea 205 has been obtained. Under comparable conditions no cross-linking was observed for this compound during a period of 6 h. This observation supports the hypothesis that labilization of chlorine atom is required for alkylation and completion of a cross-link as suggested in Chapter III. While labilization of the carbon-chlorine bond in chloroethylated bases, resulting from chloroethyl nitrosoureas, is not well understood, clearly labilization of the carbon-chlorine bond in the sulfur half-mustard can occur through a sulfonium ion (Fig. 42). Similar activation is not expected when the sulfur is replaced with oxygen. The nitrogen analogue 206, whose synthesis has thus far remained elusive, would be of interest as a potential cross-linking agent and anti-leukemic drug.



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Experimental

Materials

2-Chloroethyl-p-toluenesulfonate 183 was purchased from Eastman, 2-chloroethylmethanesulfonate 184 and tris(2-chloroethyl)phosphate 185 from Aldrich. Compounds prepared in this laboratory are described below.

The N_2O_4 used in this work was prepared by condensation of nitrogen dioxide in an appropriate flask. Oxygen was bubbled through the liquid to oxidize nitrous oxide impurities. The liquid was then distilled from P_2O_5 and stored in a sealed container at -78°C when not in use.

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosothiourea 180.

To a suspension of 2.1 g (18 mmol) of 2-chloroethylamine hydrochloride and 2.6 g (18 mmol) of cyclohexylisothiocyanate in 50 ml of chloroform at 0°C was added 2.0 g (20 mmol) of triethylamine dropwise during 30 min. After stirring an additional hour at 0°C the chloroform was removed. The white solid was suspended in water to remove hydrochloride salts filtered and air dried. The crude 1-(2-chloroethyl)-3-cyclohexylthiourea although not analytically pure was suitable for nitrosation.

To 330 mg (1.5 mmol) of the crude urea in 10 ml 98% formic acid at 0°C was added during 30 min 1.0 g (15 mmol) sodium nitrite. The mixture stirred for 2 h at 0°C and then 20 ml of water was cautiously added. After stirring an additional hour the pale yellow solid was filtered and recrystallized from ether/pet. ether. 300 mg (81% yield) m.p. 83-84°C.

Anal. Calcd. for $C_9H_{16}ClN_3OS$: C, 43.29; H, 6.41; N, 16.83. Found: C, 43.41; H, 6.48; N, 16.48. Pmr ($CDCl_3$) δ 1.0-2.2 (m, 10H, CH_2); 4.2 (t, 2H, CH_2); 6.9 (d,

1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3400 (N-H), 1700 (C=S), 1480 N=O) cm^{-1}.

2-Chloroethyl Trifluoromethanesulfonate 181.

A mixture of 6.5 g (23 mmole) of trifluoromethanesulfonic anhydride and 1.85 g (23 mmole) of 2-chloroethanol was carefully protected from moisture and heated at 60-70° for 30 min. The reaction mixture was cooled then fractionated under reduced pressure with the main fraction distilling at 58-59°/14 mm. The resulting 2-chloroethyl trifluoromethanesulfonate is extremely water sensitive and fumes readily in moist air.

Mass spectral data: Calculated for $C_3H_4ClF_3O_3S$ 176.9833 $M^+ - Cl$; 162.9676 $M^+ - CH_2Cl$; 142.9562 $M^+ - CF_3$. Found 176.9863, 162.9676, and 142.9566. Pmr (neat) δ 3.26 (t, 2H, CH_2), 4.21, (t, 2H, CH_2). Ir ν_{max} (film) 1410, 1140 (SO_2) 1200 cm^{-1} (C-F).

2-Chloroethyl p-Nitrobenzenesulfonate 182.

This compound was prepared according to the method of Reutov *et al.*[185] and isolated as white needles from benzene:cyclohexane 2.4 g (78% yield) m.p. 100-101° (lit.[185] m.p. 102-103°). Pmr ($CDCl_3$) δ 3.7 (t, 2H, CH_2); 4.4 (t, 2H, CH_2); 8.1 (d, 2H, ArH); 8.4 (d, 2H, ArH).

N-(2-Chloroethyl)-N'-nitro-N-nitrosoguanidine 186.

This compound was prepared according to the method of McKay and Milks.[187] 320 mg (61% yield) m.p. 94-96d

(lit.[187] 96d). Pmr (DMSO-d_6) δ 3.5 (t, 2H, CH_2); 4.2 (t, 2H, CH_2); 7.9 (s, 1H, exchangeable); 8.6 (s, 1H, exchangeable).

N-(2-chloroethyl)-N-nitrosoacetamide 187.

To 1.5 g (12 mmoles) of N-(2-chloroethyl)acetamide[192] in 20 ml of ether containing a suspension of 1.7 g (20 mmoles) of sodium bicarbonate at -30°C was added 2.0 g (20 mmoles) of N_2O_4 in 5 ml of ether dropwise with a syringe. The mixture stirred 1.5 h <-20°C and was then poured into 50 ml of 10% sodium bicarbonate. The ether layer was washed twice with water, dried ($MgSO_4$) and the solvent removed resulting in a dark yellow oil 1.2 g (yield 66%).

Anal. Calcd. for $C_4H_7N_2O_2Cl$ (m.w. 150.0197): C, 31.89; H, 4.70; N, 18.61. Found (150.0201, mass spectrum): C, 32.00; H, 4.70; N, 18.37. Pmr ($CDCl_3$) δ 2.80 (s, 3H, CH_3); 3.45 (t, 2H, CH_2); 4.10 (t, 2H, CH_2). Ir ν_{max} ($CHCl_3$) 1730 (C=O); 1510 (N=O) cm^{-1}.

5-[3-(2-Chloroethyl)triazenyl]imidazole-4-carboxamide 188.

This compound was prepared according to the method of Shealy *et al.*[188] and isolated as an off white solid. 45 mg (approximately 50% yield) m.p. 111-113° (lit.[188] 114°). Ir ν_{max} (nujol) 3450, 3050, 1635, 1585, 1420.

Ethyl N-(2-chloroethyl)-N-nitrosocarbamate 189.

To 1.5 g (8 mmoles) ethyl N-(2-chloroethyl)carbamate[193] in 20 ml of ether containing a suspension of 1.7 g (20 mmoles) of sodium bicarbonate at -30°C was added 2.0 g (20 mmoles) of N_2O_4 in 5 ml of ether dropwise with a syringe. The mixture stirred 1.5 h <-20°C and was then poured into 50 ml of 10% sodium bicarbonate. The ether layer was washed twice with water, dried ($MgSO_4$) and the solvent removed resulting in a pale yellow oil 1.16 g (yield 66%).

Anal. Calcd. for $C_5H_9N_2O_3Cl$ (m.w. 180.0302): C, 33.23; H, 5.03; N, 15.52. Found (180.0309 mass spectrum): C, 33.12; H, 5.01; N, 15.52. Pmr ($CDCl_3$) δ 1.45 (t, 3H, CH_3); 3.45 (t, 2H, CH_2); 4.1 (t, 2H, CH_2); 4.55 (q, 2H, CH_2). Ir ν_{max} $CHCl_3$ 1750 (C=O); 1520 (N=O) cm^{-1}.

N-(2-Chloroethyl)-N-nitroso-p-toluene sulfonamide 190.

This compound was prepared according to the method of Goodman *et al.*[2] 240 mg (52% yield), m.p. 49-50°C (lit.[2] 49-50°C). Pmr ($CDCl_3$) δ 2.4 (s, 3H, CH_3), 3.4 (t, 2H, CH_2); 4.0 (t, 2H, CH_2); 7.3 (d, 2H, ArH); 7.8 (d, 2H, ArH).

3,3'-Bis[N-(2-chloroethyl)carbamoyl]propyldisulfide 193.

500 mg (3 mmoles) of 3-hydroxypropyl disulfide[194] and 600 mg (6 mmoles) of 2-chloroethyl isocyanate were refluxed in 50 ml of ether for 6 h. After cooling the

white solid was collected and recrystallized from $CHCl_3$/pet. ether. 520 mg (yield 50%) m.p. 82-84°.

Anal. Calcd. for $C_{12}H_{22}Cl_2N_2O_4S_2$ (m.w. 392.0390): C, 36.64; H, 5.65; N, 7.12; Cl, 18.02; S, 16.30. Found (392.0389 mass spectrum): C, 36.75; H, 5.63; N, 7.07; Cl, 18.04; S, 16.12. Pmr ($CDCl_3$) δ 2.0 (m, 4H, CH_2); 2.7 (t, 4H, CH_2); 3.6 (m, 8H, CH_2); 4.2 (t, 4H, CH_2); 5.3 (s, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3340 (N-H); 1690 (C=O) cm^{-1}.

3,3'-Bis[N-(2-chloroethyl)-N-nitrosocarbamoyl]propyldisulfide 194.

To 200 mg (0.5 mmoles) of 3,3'-bis[N-(2-chloroethyl)-carbamoyl]propyldisulfide in 20 ml of tetrahydrofuran containing a suspension of 500 mg (6 mmoles) of sodium bicarbonate at -30°C was added 500 mg (6 mmoles) of N_2O_4 in 5 ml of ether dropwise with a syringe. The mixture stirred 1.5 h below -20°C. 20 ml of ether was then added and the resulting mixture poured into 50 ml of 10% sodium bicarbonate. The ether layer was washed twice with water, dried ($MgSO_4$) and the solvent removed resulting in 120 mg (yield 53%) of a yellow oil which was difficult to purify.

Anal. Calcd. for $C_{12}H_{20}Cl_2N_4O_6S_2$ (m.w. 450.0201): C, 31.94; H, 4.48; N, 12.42; Cl, 15.71. Found (450.0209, mass spectrum): C, 31.86; H, 4.51; N, 11.61; Cl, 15.51.

Pmr ($CDCl_3$) δ 2.2 (m, 4H, CH_2); 2.8 (t, 4H, CH_2); 3.4 (t, 4H, CH_2); 4.1 (t, 4H, CH_2). Ir ν_{max} ($CHCl_3$) 1750 (C=O); 1520 (N=O) cm^{-1}.

Detection of 1,3-oxathian-2-one 198.

A 3.0 ml solution was prepared containing 150 mM 3,3'-bis[N-(2-chloroethyl)-N-nitrosocarbamoyl]propyldisulfide, 200 mM potassium phosphate pH 7.3 and 0.5 M sodium dithionite in a 20% acetonitrile aqueous solution which was incubated at 37°C for 6 h. Extraction of the aqueous mixture with ether, drying ($MgSO_4$) and removal of the solvent resulted in approximately 5 mg of a colorless liquid. Although not analytically pure the mass spectral characteristics are as follows.

m/e measured	calculated	rel. intensity	fragment
118.0085	118.0089	1.05	$C_4H_6O_2S(M^+)$
74.0196	74.0190	100.00	$C_3H_6S(M^+-CO_2)$
58.0435	58.0418	13.03	$C_3H_6O(M^+-COS)$

1-[2-[(2-Chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea 199.

Triethylamine (300 mg, 3.0 mmol) was added to 500 mg (2.9 mmole) of S-(2-chloroethyl)thioethylamine hydrochloride[195] at 0-5°C in chloroform solution. Cyclohexylisocyanate (350 mg, 2.9 mmol) was added dropwise to this mixture and stirring continued at room temperature for

18 h. The chloroform was removed *in vacuo* and the residual white solid was suspended in cold water to remove hydrochloride salts and filtered. The 1-[2-[(2-chloroethyl)thio]ethyl]-3-cyclohexylurea was purified by recrystallization from chloroform/pet. ether; 450 mg (63% yield) m.p. 122-124°.

Anal. Calcd. for $C_{11}H_{21}ClN_2OS$ (m.w. 228.1296, M-36): C, 50.04; H, 8.03; N, 10.61; Cl, 13.43; S, 12.12. Found (228.1296, M-36, mass spectrum): C, 50.04; H, 8.02; N, 10.68; Cl, 13.45; S, 12.15. Pmr ($CDCl_3$) δ 0.9-2.1 (m, 10H, CH_2); 2.7 (t, 2H, CH_2); 2.9 (t, 2H, CH_2); 3.4 (t, 2H, CH_2); 3.6 (t, 2H, CH_2); 3.3-3.7 (m, 1H, CH); 4.1-4.9 (m, 2H, exchangeable). Ir ν_{max} ($CHCl_3$) 3300 (NH); 1620 cm^{-1} (C=O).

To a 100 mg (0.4 mmole) portion of the urea in 2 ml of 98% formic acid was added 200 mg (2.9 mmole) of sodium nitrite in portions during 1 h. After the addition the mixture stirred an additional hour and was then extracted with chloroform dried and the solvent removed affording 1-[2-[(2-chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea as a yellow oil, 65 mg (59% yield).

Anal. Calcd. for $C_{11}H_{20}ClN_3O_2S$ (m.w. 293.0964): C, 44.96; H, 6.87; N, 14.30; Cl, 12.06; S, 10.91. Found (293.0958, mass spectrum): C, 44.72; H, 6.76; N, 14.15; Cl, 12.33; S, 11.11. Pmr ($CDCl_3$) δ 1.0-2.2 (m, 10H, CH_2); 2.6 (t, 2H, CH_2); 2.9 (t, 2H, CH_2); 3.6 (t, 2H, CH_2);

4.0 (t, 2H, CH_2); 3.7-4.1 (m, 1H, CH); 6.8 (d, 1H, exchangeable). Ir ν_{max} ($CHCl_3$): 3350 (NH); 1725 (C=O); 1525 cm^{-1} (N=O).

1-[2-(2-Chloroethoxy)ethyl]-3-cyclohexyl-1-nitrosourea 205.

1.0 g (10 mmoles) of diethyleneglycolamine in 50 ml of 1,2-dichloroethane is saturated with HCl. The solution is cooled and 2.0 ml (27 mmoles) of thionyl chloride is added. The mixture is slowly warmed to room temperature and then heated at 60°C for 1 h. Cooling of the solution and addition of 25 ml of ether resulted in a white solid which could be recrystallized from ethanol/ether. The white crystals are hygroscopic and difficult to prepare in analytically pure form but the recrystallized 2-(2-chloroethoxy)ethylamine hydrochloride was found suitable for the next step.

To 500 mg (3 mmoles) of the hydrochloride salt and 400 mg (3 mmoles) of cyclohexylisocyanate in 50 ml of chloroform at 0°C is added 300 mg (3 mmoles) of triethylamine during a 30 min period. After stirring 2 h at 0°C the solution was warmed to room temperature and stirred another 2 h. Removal of the solvent resulted in a white solid which was suspended in water and stirred to remove hydrochloride salts. The remaining white solid was filtered and taken up in chloroform, dried and crystallized by adding pet. ether 480 mg (yield 65%) m.p. 82-84°.

Anal. Calcd. for $C_{11}H_{21}ClN_2O_2$ (m.w. 248.1291): C, 53.10; H, 8.53; N, 11.26. Found (248.1305, mass spectrum): C, 53.11; H, 8.74; N, 11.09. Pmr ($CDCl_3$) δ 1.0-2.1 (m, 10H, CH_2); 3.3-3.8 (m, 9H, CH_2); 4.5 (d, 1H, exchangeable); 4.8 (t, 1H, exchangeable). Ir ν_{max} ($CHCl_3$) 3200 (N-H); 1630 (C=O) cm^{-1}.

To 100 mg (0.4 mmol) of the urea in 1 ml of 98% formic acid at 0°C is added during 20 min 100 mg (1.4 mmol) sodium nitrite. After the addition the mixture is stirred 1 h, 0°C then 5 ml of H_2O is cautiously added. The aqueous mixture is extracted with chloroform, washed, dried ($MgSO_4$) and the chloroform removed to yield 60 mg (yield 54%) of a yellow oil which was difficult to purify.

Anal. Calcd. for $C_{11}H_{20}ClN_3O_3$: C, 47.50; H, 7.27; N, 15.13. Found: C, 46.61; H, 7.15; N, 14.69. Pmr ($CDCl_3$) δ 1.0-2.2 (m, 10H, CH_2); 3.5 (m, 6H, CH_2); 3.9 (m, 1H, CH); 4.1 (t, 2H, CH_2). Ir ν_{max} ($CHCl_3$) 3400 (N-N); 1720 (C=O); 1520 (N=O) cm^{-1}.

Although an acceptable measurement could be made for the parent compound, the high resolution mass spectrum was characterized by a tendency to undergo proton transfer to form cyclohexyl isocyanate and the appropriate diazohydroxide. Mass spectral data:

Measured	Calculated	%rel. intensity	fragment
277.1191	277.1193	1	M^+
150.0350	150.0352	24	$ClCH_2CH_2OCH_2CH_2N_2OH^+$
125.0822	125.0840	2	$C_6H_{11}NCO^+$
109.0207	109.0235	31	$ClCH_2CH_2OCH_2CH_2^+$
93.0100	93.0107	21	$ClCH_2CH_2OCH_2^+$
83.0873	83.0861	100	$C_6H_{11}^+$

Methods

In vivo testing was done by Mr. I. Wodinsky, Arthur D. Little, Inc. under the direction of the National Cancer Institute, Silver Spring, Maryland. Testing was in L1210 inoculated mice with a single intraperitoneal injection of drug. Values are reported as % (T/C) which is: (the life span of L1210 inoculated mice treated with drug divided by the life span of L1210 inoculated mice given no drug) x 100.

SUMMARY

This study has examined a number of aspects concerning the chemistry of 2-haloethylnitrosoureas. Polarography has allowed a convenient measurement of the stabilities of the nitrosoureas under aqueous physiologically buffered solution. Decomposition studies have confirmed the existence of the 2-chloroethyl alkylating agent for chloroethylnitrosoureas resulting from a major decomposition pathway. Evidence was presented which also suggests

the intermediacy of an oxadiazoline in the decomposition of some nitrosoureas to account for the isolated carbonyl containing compounds and hydroxyethylated nucleophiles.

Details concerning the reactions of nitrosoureas with purified DNA have also been presented. In addition to alkylation some nitrosoureas produce DNA interstrand cross-links. This phenomenon is most apparent with 2-chloroethylnitrosoureas in accord with observed anti-leukemic properties. Cross-linking appears to result from chloroethylation of an appropriate base followed by labilization of the carbon-chlorine bond and a second alkylation involving displacement of chloride ion. The observation that the extent of DNA interstrand cross-linking produced by chloroethylnitrosoureas is less than 50% can be accounted for by three processes: (i) low levels of DNA alkylation; (ii) competing intramolecular alkylation after chloroethylation; and (iii) concomitant DNA degradation.

DNA degradation has also been examined in detail. Nitrosourea induced single strand scission (SSS) occurs by two major processes. Type I SSS results from phosphate alkylation and is extensive in the case of hydroxyethyl alkylating agents. Type II SSS results from alkylation of the bases followed by depurination or depyrymidination. Conversion of the apurinic site to a single strand break can occur enzymatically, under high

pH conditions or by reaction with an appropriate amine.

The final aspect presented in this study involved the design of new drugs based on the results of the previous chapters. The two most productive design areas involved attempts to generate chloroethyl alkylating agents from sources other than nitrosoureas, and the modification of the alkylating portion of CCNU to enhance DNA interstrand cross-linking. The extent of cross-linking of these compounds was observed to correlate with *in vivo* antileukemic data.

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